FUNCTIONAL GENOMICS OF BACTERIOCINOGENIC LACTIC ACID BACTERIA FROM SPROUTS AND APPLICATION AS BIOLOGICAL CONTROL AGENTS

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Fresh sprouts have been responsible for 30 foodborne illness outbreaks and 1800 cases since 1996 in the United States. Use of chemical sanitizers prior to the sprouting process has been attempted in many studies to decontaminate or control the growth of pathogens. In 1999, FDA issued a guidance to reduce microbial food safety hazards for sprouted seeds. However, even after FDA's guidance, continuous outbreaks have still been a serious concern for consumers.

In an attempt to control the growth of foodborne pathogens on sprouts using protective cultures, lactic acid bacteria (LAB) were isolated from fermented sprouts. Many of the LAB isolates were found to produce bacteriocins, which are ribosomally synthesized antimicrobial peptides that can inhibit closely related bacteria. One bacteriocin, designated mundticin L, produced by *Enterococcus mundtii* CUGF08 was found to inhibit *Listeria* spp. and other genera. Another bacteriocin, produced by *Lactococcus lactis* AA4 was found to be nisin Z. The genetic determinants for mundticin L, the associated ATP-binding cassette (ABC) transporter, and immunity protein were identified, cloned, and sequenced. Mundticin L was isolated and confirmed by N-terminal amino acid sequencing. The function of the immunity protein was analyzed by heterologous expression of the immunity gene in sensitive Gram-positive organisms. The immunity protein does not provide full protection.

However, additional factors may be involved in this unusual phenotype. Genomic mutagenesis may reveal these factors.

Thermal inactivation and biological control utilizing naturally occurring bacteriocinogenic LAB were investigated as potential decontamination strategies. Both methods showed potential in enhancing the safety of fresh sprouts. LAB as bioprotective cultures to enhance the safety of sprouts may serve a model as a "natural" or "green" hurdle for the safety of non-fermented produce.

BIOGRAPHICAL SKETCH

Guoping Feng was born to Xinhua Shen and Deming Feng in 1979 and raised in the eastern province of Zhejiang, China. He and his younger sister Guofen Feng grew up in the countryside of Tongxiang County where they had a happy and memorable childhood.

He received his B.S. degree in food science and engineering in 2002 from Southwest Agricultural University in Chongqing, where he met his wife Qun Sun in the same class during the fall of 1998. He then moved to Shanghai to further his education with a M.S. in food science at Jiao Tong University. He wanted to go to the United States for a quality education of his prospective Ph.D. program. After graduation with outstanding academic achievements in 2005, he was awarded the Vitasoy and Lo Fellowship by Cornell University to pursue his Ph.D. degree. He was interested in food microbiology and started his Ph.D. program with Dr. Randy W. Worobo, an expert in antimicrobial peptides and microbial food safety.

After earning his Ph.D., he will continue his academic career as a postdoctoral researcher at the University of Rochester School of Medicine and Dentistry.

To my mother Xinhua Shen, father Deming Feng,

sister Guofen Feng

and beloved wife Qun Sun

献给母亲沈新华、父亲冯德明

妹妹冯国芬

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CHAPTER 1

INTRODUCTION

FOODBORNE ILLNESSES ASSOCIATED WITH FRESH PRODUCE

Fruits and vegetables are an integral part of a daily diet. Besides their palatable taste, they may reduce the risks of chronic diseases including strokes, type 2 diabetes, some types of cancer, and possibly cardiovascular disease, and hypertension (16). They are usually low in calories and high in dietary fiber, vitamins and antioxidants, among other nutrients. The consumption of fresh produce has been increasing with the rising consumer awareness of health.

However, consumption of fresh produce has raised a public health issue due to a series of foodborne illness outbreaks which implicated fresh produce as a vehicle for human pathogens. The number of illness outbreaks associated with fresh produce has been increasing. During 1973-1997, the proportion of produce-associated illness outbreaks in the United States increased from 0.7% in the 1970s to 6% in the 1990s (106). During the period of 1990-2005, the proportion of produce-associated outbreaks increased to the highest level at 13% (36). In addition, the total number of produce-associated outbreaks has also increased. The increase in the number of outbreaks due to the consumption of fresh produce may be attributed to the following reasons: the rising consumption of fruits and vegetables during the past decades; the changing pattern in production and distribution of fresh produce, especially the globalization of the produce market with relatively localized strictness of agricultural and manufacturing practices; and the enhanced surveillance system for foodborne diseases, especially in the United States and Europe, which resulted in higher reported numbers of outbreaks than before (47).

During 1973-1997, *Salmonella* spp. was the most common pathogen associated with produce, followed by *E. coli* O157:H7, *Shigella*, and *Campylobacter*, while the most common virus was hepatitis A followed by norovirus. *Cyclospora cayetanensis* was the most frequent foodborne parasite associated with produce (106). However, during the period of 1990-2005, the most prevalent foodborne illness was attributed to norovirus infection while *Salmonella* spp. and *E. coli* O157:H7 remained as the primary etiological bacterial foodborne pathogens associated with foodborne illness (36).

Salad, mixed fruits and vegetables accounted for approximately one third of all outbreaks and other single produce commodities were responsible for the remainder. Foodborne illness implicated single produce items include (in order of frequency for outbreaks) lettuce, melon, seed sprout, apple or orange juice, berries, tomato, green onion, carrot, apple, pear, pineapple, basil, celery, cucumber, fresh elderberry, and fresh-squeezed lemonade (106). Recently, spinach and jalepeno peppers joined this list after the notorious large multistate outbreaks due to consumption of spinach contaminated with *E. coli* O157:H7 in 2006 (20) and jalepeno peppers contaminated with *Salmonella* Saintpaul in 2008 (18).

With the increasing number of produce-associated outbreaks, the relationship between pathogens and types of produce is being recognized, although scientific research on the molecular mechanism of how certain pathogens attach or colonize to specific produce has just started. Pathogen-food pairs are established based on the history of produce-associated outbreaks; for example, *Salmonella* spp. from sprouts, melons, tomatoes, peppers, orange juice; *E. coli* O157:H7 from leafy greens, apple juice/cider and sprouts. The relationship between pathogens and produce is influenced by many factors, including the nature of the surface for attachment/colonization and the bacterial strain itself. For example, a recent genomic analysis of the *E. coli*

O157:H7 strain that was associated with the 2006 spinach outbreak revealed a unique DNA fragment that contains genes for enhanced virulence and adaptation to plants (75). However, no conclusive mechanism has been established to elucidate the colonization of human pathogens on plant surfaces although it is recognized that strains even within the same species vary in their ability to colonize plant surfaces and pathogens can be internalized into the tissue of fresh produce which renders surface disinfectants unreliable for eliminating pathogens (117).

The source of contamination depends on the specific pathogen. *Salmonella* spp., *E. coli* O157:H7, *Shigella* spp. and *Campylobacter* spp. are natural inhabitants in animal or human hosts. For *Salmonella* spp. and *Campylobacter* spp., avians are the primary reservoir and vehicle, while for *E. coli* O157:H7, bovine are the most common carriers. Humans are the natural reservoir for *Shigella* spp. Fresh produce can be contaminated with foodborne pathogens at any point in the chain from farm to table. Therefore, it is not surprising that waste and manure shed from domestic or wild animals in the field is a dominant route for *E. coli* O157:H7 and *Salmonella* spp. contamination while the majority of shigellosis outbreaks have been attributed to direct fecal contamination by food handlers with poor hygiene practices. Post-harvest cross-contamination in food processing plants and food catering kitchens are additional potential risks for all foodborne pathogens.

Although various factors as mentioned above could play a role in the emerging illness outbreaks associated with produce consumption, pre-harvest contamination in the field has been the primary cause. The initial microbial load on produce is due to pre-harvest contamination (pathogenic and non-pathogenic microorganisms) during growing. The initial microbial load on the produce influences how effective the downstream (post-harvest) antimicrobial processing and treatments will be. Once contaminated animal manure or runoff from livestock farm is released to the

environment, *E. coli* O157:H7 and *Salmonella* spp. may survive in soil for months (48, 49, 104). Moreover, the rhizosphere of plants in soil was found to enhance the survival of *E. coli* O157:H7 for even longer times (49, 59). The surviving pathogens can contaminate fresh crops directly or indirectly. Pathogens from untreated manure can contaminate irrigation water which in turn may contaminate the rhizosphere or phyllosphere of the growing plants.

A systematic approach is needed to control pre-harvest and post-harvest contamination. This approach requires involvement of research institutions and regulatory agencies to assess potential alleviation or decontamination methods and incorporate these novel methods into regulatory intervention programs such as Good Agriculture Practices (GAP), Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Control Points (HACCP) to protect the food supply. The effectiveness of these process control strategies has been proven for specific food commodities. For example, the number of outbreaks associated with juice has been significantly reduced according to CDC reports since FDA implemented the Juice HACCP regulation (CFR 120.20) that required a juice HACCP plan and the 5-log reduction performance standard (120).

A SPECIAL CONCERN WITH ALFALFA SPROUTS AND INTERVENTION METHODS FOR DECONTAMINATION

Fresh sprouts are considered as a healthy food that is low in calories and rich in dietary fiber and phytoestrogens that may prevent menopausal symptoms, osteoporosis, cancer and heart disease (76). Fresh sprouts are widely consumed raw or lightly cooked as in salads, sandwiches, hamburgers, etc. However, they are notorious for being a food vehicle for human pathogens such as *Salmonella* spp. and *E. coli* O157:H7. Table 1.1 is an updated list of foodborne illness outbreaks associated with fresh sprouts.

Table 1.1. List of foodborne illness outbreaks associated with fresh sprouts.

Year	Pathogen	Cases	Country	Type of sprout
1976	*Bacillus cereus*	4	U.S.	Vegetable
1982	*Y. enterocolitica*	16	U.S.	Mung bean
1988	*S.* Saintpaul, *S.* Havana, *S.* Muenchen	148	Sweden	Mung bean
1988	*S.* Saintpaul, *S.* Virchow	150	U.K.	Mung bean
1989	*S.* Gold-Coast	31	U.K.	Cress
1990	*S.* Anatum	15	U.S.	Alfalfa
1992	*S. enterica*	272	Finland	Alfalfa
1994	*S.* Bovismordificans	492	Finland, Sweden	Alfalfa
1995	*S.* Stanley	242	Finland, U.S.	Alfalfa
1995	*S.* Newport	154	Denmark	Alfalfa
1995/96	*S.* Newport	133	U.S., Canada, Denmark	Alfalfa
1996	*E. coli* O157:H7	5,853	Japan	Radish
1996	*S.* Montevideo and *S.* Meleagridis	492	U.S.	Alfalfa
1997	*S.* Anatum and *S.* Infantis	109	U.S.	Alfalfa
1997	*E. coli* O157:H7	187	U.S.	Alfalfa
1997	*S.* Meleagridis	78	Canada	Alfalfa
1997/98	*S.* Senftenberg	60	U.S.	Alfalfa
1998	*S.* Havana and *S.* Cubana	40	U.S.	Alfalfa
1998	*E. coli* O157:H7	8	U.S.	Alfalfa, clover
1999	S. Mbandaka, *S.* Muenchen, S. Typhimurium	>352	U.S.	Alfalfa
1999	*S.* Saintpaul	36	U.S.	Clover
1999	*S. paratyphi* var Java	51	Canada	Alfalfa
2000	*Salmonella* spp.,	22	U.S.	Alfalfa
2000	*S.* Enteritidis PT 4b	27	Netherlands	Mung bean
2000	*S.* Enteritidis	75	U.S.	Mung bean
2001	*S.* Enteritidis PT 913	84	Canada	Mung bean
2001	*S.* Enteritidis	35	U.S.	Mung bean
2001	*S.* Kottbus	32	U.S.	Alfalfa
2002	*S.* Enteritidis	NA	U.S.	Mung bean
2002	*E. coli* O157:H7	5	U.S.	Alfalfa
2003	*E. coli* O157:H7	7	U.S.	Alfalfa
2003	*E. coli* O157:NM (H-)	13	U.S.	Alfalfa
2003	*S.* Chester, *S.* Saintpaul	42	U.S.	Alfalfa

Table 1.1 (continued)

2002	*S.* Enteritidis	NA	U.S.	Mung bean
2002	*E. coli* O157:H7	5	U.S.	Alfalfa
2003	*E. coli* O157:H7	7	U.S.	Alfalfa
2003	*E. coli* O157:NM (H-)	13	U.S.	Alfalfa
2003	*S.* Chester, *S.* Saintpaul	42	U.S.	Alfalfa
2004	*E. coli* O157:H7	2	U.S.	Alfalfa
2004	*S.* Bovismordificans	35	U.S.	alfalfa
2005	*S.* Enteritidis PT 13	552	Canada	Mung bean
2006	*Salmonella* spp.	>100	Australia	Alfalfa
2006	*S.* Bareilly or *S.* Virchow	115	Sweden	Mung bean
2006	*S.* Braenderup	4	U.S.	Bean
2007	*S.* Stanley	51	Sweden	Alfalfa
2007	*S.* Weltevreden	45	Norway, Denmark, Finland	Alfalfa
2007	*S.* Mbandaka	15	U.S.	Alfalfa
2007	*S.* Montevideo	24	U.S.	Bean
2009	*S.* Saintpaul	228	U.S.	Alfalfa

Note: This list is compiled from a summary report from Food Standards Agency (46) and other governmental or institutional sources (17, 19, 21-25, 33, 39, 43, 94, 96, 125, 126).

The majority of the outbreaks were due to *Salmonella* spp. and the most implicated type of sprouts was alfalfa sprouts. Fresh sprouts, especially alfalfa sprouts represent such a dangerous health risk for consumers, that in 1999 the FDA started to issue a warning that "those persons who wish to reduce the risk of foodborne illness from sprouts are advised not to eat raw sprouts" (118). It was a result of clustered foodborne outbreaks associated with consumption of fresh sprouts since 1995 (Table 1.1). Table 1.1 indicates that the most common pathogen for sprout illness outbreaks is *Salmonella* spp., followed by *E. coli* O157:H7 and other less frequently associated pathogens. The most commonly implicated type of sprouts is alfalfa sprouts, followed

by mung bean sprouts. *Listeria monocytogenes* has been detected in alfalfa sprouts and resulted in multiple food recalls although outbreaks linked to *Listeria monocytogenes* contamination have not been reported. One study demonstrated that *Listeria monocytogenes* can rapidly grow to high levels during the sprouting process (89). The potential risk of listeriosis from consumption of fresh sprouts can not be underestimated due to its high fatality rate and zero tolerance for *L. monocytogenes* contamination level in many countries, including the United States.

Contaminated seeds are the primary source for most sprout-associated outbreaks (82), i.e., the seeds were contaminated with *Salmonella* spp. and/or *E. coli* O157:H7 in the growing field, although contamination during production, processing, packaging, distribution and preparation may also have occurred. The contamination level present on the seeds was found to be very low. It was estimated that *Salmonella* spp. existed on naturally contaminated seeds at levels lower than 1-6 CFU/100g seeds (82). Although the number of pathogenic cells on seeds do not exceed the infective dose for *Salmonella* spp. and *E. coli* O157:H7, the conditions used for sprouting (~25°C for 3 or more days with humidity and nutrients released from young sprouts) provide an optimal incubation for the growth of bacterial foodborne pathogens. The pathogenic cells on naturally contaminated seeds can grow to high levels (111), amplifying the risk of pathogen infection after consumption of the finished sprouts.

Due to the fact that the seeds are contaminated at the pre-harvest stage, the most feasible intervention strategy to control the risk of the outgrowth of foodborne pathogens is to decontaminate the seeds prior to sprouting so that pathogens will not have a chance to multiply during the sprouting process. However, alfalfa seeds have been found to be difficult to decontaminate due to their special seed structures, such as wrinkles and crevices on the surface of seeds, that can protect pathogens from being accessed by surface sanitizers (26). A number of disinfection methods have been

investigated in an attempt to decontaminate seeds. The diversity of the methods investigated for their potential in improving the safety of sprouted seeds was a display of most, if not all, of antimicrobial approaches that already existed in modern food microbiology. These treatments include physical, chemical and biological strategies. Physical treatments include irradiation, heat and pressure. Irradiation treatments were reported to inactivate *Salmonella* spp. and *E. coli* O157:H7 by less than 5 logs; however, irradiation reduced the germination and yield of sprouts (for example, length of sprouts) especially when the dose exceeded 2 kGy (6, 97-99, 102, 116). Heat treatment has an advantage of being able to inactivate pathogens trapped in crevices and winkles which are physical barriers for chemical sanitizers. Wet heat treatment at high temperatures for relatively short times as a sole treatment was found to be effective in achieving a 5-log reduction on seed types other than alfalfa seeds without affecting seed viability, but the viability of alfalfa seeds was more easily reduced (5, 121, 122). It is known that seeds can tolerate dry heat treatment which has been shown to be a promising approach for mung bean seeds (57). High hydrostatic pressure especially in combination with mild heat was reported to be effective in eliminating 5-logs of *E. coli* O157:H7 populations on alfalfa seeds without significantly affecting seed viability (83-85). Chemical treatments of seeds using hypochlorite, trisodium phosphate, chlorine dioxide, hydrogen peroxide, calcium hydroxide, lactic acid, citric acid, acetic acid, fatty acids, allyl isothiocyanate, ammonium (9-11, 55, 61, 90, 92, 103, 115, 123, 124) have been investigated for their effectiveness in decontamination of experimentally inoculated alfalfa seeds. However, none of these methods were capable of achieving a 5-log reduction in pathogen populations without negatively affecting seed viability or causing organoleptic defects in the finished sprouts. *Salmonella* spp. and *E. coli* O157:H7 commonly have been recovered or detected after treatments by enrichment culturing, suggesting that elimination of pathogens in seeds,

in order to prevent multiplication of surviving cells, is not possible by these chemical treatments. Among these chemicals, hypochlorite attracted attention for its efficacy in reducing pathogens on seeds without reducing seed viability. In 1999, after a cluster of foodborne illness outbreaks over a 3-year period, FDA issued two guidance documents to the sprout industry: "Reducing Microbial Food Safety Hazards for Sprouted Seeds" and "Sampling and Microbial Testing of Spent Irrigation Water during Sprout Production". The former recommends that seeds be soaked in 20,000 ppm calcium hypochlorite for 15 min as a single treatment step prior to sprouting while the latter advises that spent irrigation water be sampled for microbial testing as an indicator for contamination of sprouts.

Unfortunately, outbreaks continued to occur after FDA's recommendations. Investigations revealed that outbreaks were linked to sprouted seeds that were pretreated following FDA's guidance (95). Based on subsequent outbreaks, it is evident that a single-step treatment with surface sanitizer can not sufficiently eliminate pathogens in seeds, and thus prevent the outgrowth of pathogens during sprouting.

New intervention methods that are capable of eliminating pathogens in seeds and/or control the growth of pathogens during sprouting are needed to enhance the safety of alfalfa sprouts. As mentioned above, thermal inactivation may have potential due to its effectiveness regardless of seed structure. Biological control utilizing protective cultures, particularly food-grade lactic acid bacteria, may be another promising strategy to inhibit the growth of pathogens during sprouting.

LACTIC ACID BACTERIA AS BIOLOGICAL CONTROL AGENTS

Lactic acid bacteria (LAB) are a diverse group of Gram-positive, non-sporulating and non-respiring cocci or rods whose main end product of metabolism is lactic acid (101). The delimitation of lactic acid bacteria has been a controversy since some genera such as *Listeria* and *Bacillus* that are not traditionally considered as LAB can

produce lactic acid. However, it is generally agreed that the LAB group includes *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, *Pediococcus*, *Oenococcus, Aerococcus*, *Carnobacterium*, *Tetragenococcus*, *Weisella* and *Vagococcus* (113). The genomes of LAB are mostly AT-rich and contain less than 50% GC (129). Lactic fermentation by LAB started to be an integral part of food production in the history even before humans recognized their role. Besides the flavors LAB fermentations afford, LAB fermentation serves as a preservation method for foods that otherwise would be perishable within a shorter shelf life. LAB are known as starter cultures in fermentations to produce milk products such as yogurt and cheese, meat products such as sausage and hams, vegetables and fruits such as sauerkraut and pickles and wines. Members of LAB have been identified to be probiotics that are "live microorganisms, which when consumed in adequate amount, confer a health benefit on the host" (93). The health benefits include alleviation of disorders in gastrointestinal tract, improved immune system and prevention of certain chronic disease and cancer although the molecular mechanisms for those benefits were not clear (93).

The protective properties of LAB in foods have been recognized and attracted great attention from scientists. With consumers' rising dietary awareness and their demand for more "natural" or "green" technologies used in food processing, chemical preservatives have become a public concern due to their variable toxicity and side effects to humans. The increasing consumption of fresh produce and minimally processed foods challenges conventional preservation methods, and the use of non-thermal and natural strategies as alternative preserving methods has been examined for their feasibility to meet consumer demands while guaranteeing food safety. LAB produce lactic acid and other organic acids, reuterin, hydrogen peroxide, and bacteriocins which are ribosomally synthesized peptides that can inhibit closely

related bacteria (66). Besides the antimicrobial compounds that LAB produce, the growth of LAB themselves is a competitive exclusion of other spoilage and pathogenic microflora that otherwise may grow to high levels, resulting in shorter shelf life or foodborne illness. LAB may be directly applied into food systems without approval from FDA because the majority of LAB are generally recognized as safe (GRAS). Because of the GRAS status, LAB and their metabolites are all considered to be "green, safe and natural". However, purified compounds such as bacteriocins that are produced by LAB are subject to FDA approval because it is considered as an additive. Therefore, application of live LAB cultures rather than their metabolites in food systems to extend shelf life and improve food safety is a promising alternative without extensive regulatory involvement for application.

Many studies in biopreservation using LAB have been directed at controlling pathogens in meat products, especially vacuum-packed meats, sausages, raw meats and cooked meats. *Listeria monocytogenes* is the primary pathogen of concern in cooked meats, whereas post-processing contamination by *Listeria monocytogenes* has been responsible for numerous outbreaks. Sausages pose a food safety concern with contamination and survival of *Listeria monocytogenes* since they are not cooked and *Listeria monocytogenes* is capable of growing at refrigeration temperatures. Another pathogen that poses food safety concern especially in vacuum-packed or modified-atmosphere-packed products is *Clostridium botulinum*, which can produce deadly neurotoxins in foods. Therefore, many studies have investigated the use of LAB to control these two pathogens in meat products. *E. coli* O157:H7 and *Salmonella* spp. are also potential pathogens with meat and meat products but fewer studies using LAB as biocontrol cultures have been performed. Since LAB are part of the natural microflora in meats and even dominate certain foods, screening for protective strains from their natural microbiota is a good strategy (112). Criteria for screening include

their growth rate in select food systems, acid production, bacteriocin production and their inhibitory activity against target pathogens or spoilage organisms. Bacteriocinogenic LAB have attracted much attention due to their inhibitory activity against *Listeria monocytogenes*, in addition to antimicrobial effects of decreased pH, hydrogen peroxide and competition for growth. Nisin-producing *Lactococcus lactis* subsp. *lactis* has been extensively studied because nisin can inhibit both *Listeria monocytogenes* and *Clostridium botulinum* while pediocin-producing *Pediococcus acidilactici* was used to inhibit *Listeria monocytogenes* due to the higher antimicrobial activity of pediocin. In general, bacteriocinogenic LAB were shown to be promising candidates for controlling the growth of pathogens such as *Listeria monocytogenes* and *Clostridium botulinum*. Inhibition of Gram-negative pathogens such as *E. coli* O157:H7 and *Salmonella* spp. is primarily due to the acids that LAB produced and to a lesser extent, their competitive exclusion (72).

Research in biological control of pathogens in fresh produce is just in its early stages. While bacteriocinogenic LAB are known to play an important role in preserving fermented vegetables such as olives, sourdough, miso and sauerkraut, their potential role in protecting non-fermented vegetables has yet to be fully investigated (105). Non-bacteriocinogenic *Lactococcus lactis* isolated from alfalfa sprouts were studied for its inhibitory activity against *Listeria monocytogenes* on alfalfa sprouts. It was found that although *Lactococcus lactis* showed some inhibition against *Listeria monocytogenes* during sprouting compared to the control, *Listeria monocytogenes* was shown to still be able to grow into high levels (89). Cai and his co-workers isolated bacteriocin-producing *L. lactis* subsp. *lactis* and *Enterococcus faecium* strains from mung bean sprouts and applied these strain to Caesar salad to control the growth of *L. monocytogenes* (15). Both bacteriocinogenic LAB inhibited *L. monocytogenes* in salads compared to the control; however, when the salad was co-inoculated with both

strains, no synergic effect was observed. *Lactococcus lactis* displayed a stronger inhibitory effect than *E. faecium*. Bennik and co-workers (7) isolated mundticin-producing *Enterococcus mundtii* ATO6 and pediocin-producing *Pediococcus parvulus* from chicory endive. *E. mundtii* ATO6 was able to produce mundticin at refrigeration temperatures. Although both strains showed inhibition on *L. monocytogenes* in model medium systems, they did not inhibit the growth of *L. monocytogenes* on fresh mung bean sprouts stored under modified atmosphere at 8°C. Few studies have investigated the potential antagonistic effect of LAB on Gram-negative pathogens such as *E. coli* O157:H7 and *Salmonella* spp. which are the pertinent pathogens for sprout-associated outbreaks. *L. lactis* subsp. *lactis* and *Pediococcus acidilactici* isolated from alfalfa seeds were tested for their inhibition on *Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes* (127). Both strains demonstrated significant antagonistic properties against these pathogens; unfortunately, the study was performed in MRS broth rather than fresh sprouts. *Pseudomonas fluorescens* 2-79 was shown to effectively inhibit the growth of *Salmonella* spp. during sprouting of alfalfa seeds (44). Although *Pseudomonas* spp. are known members of the indigenous microflora on fresh sprouts, artificial level of pseudomonads as biocontrol agents in foods may be a controversial issue due to their non-food-grade status.

AN UPDATED REVIEW OF BACTERIOCINS: THEIR CLASSIFICATION, BIOSYNTHESIS, REGULATION, IMMUNITY, RESISTANCE, MODE OF ACTION AND ANTIMICROBIAL SPECTRUM

Bacteriocins are ribosomally synthesized proteinaceous compounds that inhibit closely related bacteria (66). Although scientific attention has been directed at bacteriocins produced by Gram-positive LAB due to their promising applications in foods, “bacteriocin” was coined for colicin which was the first discovered bacteriocin from *E. coli* (30). Mankind has benefited from bacteriocins produced *in situ* by

indigenous LAB microflora since the discovery of fermented foods. However, deliberate use of bacteriocins emerged only five decades ago when nisin was first marketed in England. Nisin was officially approved for use as a food additive in 1988 by the U.S. FDA. Scientists have been focusing on isolation, characterization and application of bacteriocins in food systems. Novel bacteriocins continue to be discovered and added to the long list of known bacteriocins. Discovery of bacteriocins has been the result of mostly phenotypic observations by inhibition assays in microbial media. The advancement of nucleotide sequencing technologies has made high throughput genome-sequencing of microorganisms more achievable in terms of cost and time. Genome-wide data mining may be a powerful tool to find open reading frames that encode novel bacteriocins which would be difficult to be revealed by traditional phenotype-based assays (91). Genomics of lactic acid bacteria will accelerate the discovery of bacteriocins, especially for those whose production is conditional; more and more bacteriocins are being determined to be produced only by induction by a cell-density-dependent signal peptide (quorum sensing).

Bacteriocins, their genetic organization, biosynthesis, classification, characteristics and application in various foods have been reviewed extensively by many researchers (29, 30, 38, 41, 50, 60, 66, 86, 105, 128). Therefore, an updated, rather than a comprehensive review will be given here. Some areas which other reviews did not cover will be discussed more in depth.

Classification of bacteriocins. Bacteriocins are classified into three main classes. Klaenhammer (66) proposed that bacteriocins be divided into four distinct classes: class I, lantibiotics containing post-translationally modified amino acids such as lanthionine, β-methyllanthionine and dehydrated residues; class II, consisting of small, heat-stable and unmodified peptides; class III, bacteriocins which are large heat-labile proteins; class IV, containing complex bacteriocins which requires chemical moieties

such as lipid and carbohydrate for activity. The classification scheme changed with newly identified bacteriocins challenging the existing knowledge. Class IV as an independent group was controversial and questioned due to the lack of scientific information about these bacteriocins and to the later finding that the claimed loss in activity of the peptide moiety might be the result of purification artifact (62, 86). Cotter (30) proposed a modified scheme for three classes, with class III assigned for bacteriolysins as non-bacteriocin lytic proteins which can hydrolyze cell walls of target organisms and may not have dedicated immunity proteins. However, class III should be excluded from the bacteriocin classification, considering the limited application in foods, and the differences in molecular structure and genetic organization.

Class I bacteriocins are a group of modified peptides that are so diverse in structure, biosynthesis and amino acid sequence that sub-classification into smaller groups is difficult although some researchers proposed subclasses (38, 128). The most important structural feature is that they contain modified amino acids. Amino acids with a hydroxyl group may be dehydrated by dehydratase. For example, serine and threonine in nisin may be dehydrated into didehydroalanine (Dha) and didehydrobutyrine (Dhb), respectively. The sulfhydryl group of cysteine can react with the double bonds in Dha and Dhb to form lanthionine and β-methyllanthionine, respectively. Since the reaction may occur between cysteine and Dha/Dhb that could be distant in primary structure, the resultant modified amino acids produce structural rings which are believed to play an important role in the antimicrobial activity of lantibiotics.

Class II bacteriocins are small, heat-stable and unmodified peptides that may be divided into three or four subclasses. Klaenhammer (66) proposed that class II bacteriocins be divided into three subclasses: Class IIa containing *Listeria*-active peptides with a N-terminus of Tyr-Gly-Asn-Gly-Val-Xaa-Cys motif; class IIb of two-

peptide bacteriocins; class IIc of thio-activated peptides. This scheme did not indicate the true distinctiveness of different class II bacteriocins in terms of their primary structure, function and antimicrobial spectrum. Also, these subclasses could not accommodate all class II bacteriocins that were subsequently found. Cotter (30) proposed that class II bacteriocins be divided into four subclasses: class IIa of pediocin-like bacteriocins which is the same group proposed by Klaenhammer; class IIb of two-peptide bacteriocins; class IIc of cyclic bacteriocins; and class IId of non-pediocin linear peptides. Drider (38) divided the class II bacteriocins into three subclasses: class IIa of pediocin-like bacteriocins; class IIb of two-peptide bacteriocins; class IIc of other bacteriocins. All the schemes for classification agreed that pediocin-like bacteriocins with a conserved N-terminus of YGNGV and two-peptide bacteriocins be separate subclasses. However, opinions differed about whether the cyclic peptides should be separated from other miscellaneous peptides. With more and more class II bacteriocins being discovered, the information on the structure of bacteriocins and their function would be sufficient for a clearer and open-ended classification. For convenience, the following discussions adopt the classification scheme proposed by Cotter (30).

Genetics of biosynthesis and regulation. Production of bacteriocins requires genes for a dedicated transporter protein that may have protease activity to cleave the precursor peptide and for immunity proteins that protect the producer organisms against their own bacteriocins. The genetic organization is different depending on which class of bacteriocins. The genetic organization of class I bacteriocins is more complex than that of class II bacteriocins because class I bacteriocins require enzymes for post-translational modifications. The molecular mechanism for immunity is also more complex. Immunity to nisin requires more than one protein which is sufficient for immunity in class II bacteriocins.

The structural gene for nisin encodes a 57-residue precursor with 23 residues for its leader peptide and 34 residues for the mature bacteriocin. The primary structure of nisin is highly homologous to subtilin and epidermin (14). Eleven genes have been identified for nisin production (73, 74). These genes are clustered in the chromosome of *L. lactis*. *NisA* encodes nisin A precursor. Downstream genes following *NisA* are *NisBTCIPRKFEG*. NisB and NisC are membrane-associated proteins that are responsible for nisin modification and maturation (40). NisT is a dedicated transporter for NisA. NisP is a protease that cleaves the nisin precursor so that the mature bacteriocin is released to extracellular environment for its antimicrobial function. NisI and NisFEG are proteins believed to be involved in producer immunity to nisin. NisR and NisK are regulatory proteins for nisin biosynthesis. Nisin is an auto-regulator of its own biosynthesis. The producer organism can quorum-sense extracellular level of the fully modified and mature bacteriocin through a two-component regulatory system consisting of proteins NisK and NisR (74). The membrane-associated histidine kinase (NisK) senses extracellular nisin molecules and activates the response regulator (NisR). The response regulator can activate transcription of the nisin gene and other genes in the same operon. The nisin-inducible expression system has been attracting scientific attention due to its potential use for overproduction of proteins and nutraceuticals that have industrial and therapeutic values. Products of interest were shown to be overproduced in *L. lactis* strains on the condition of nisin being added as an inducer (34). The gene for β-glucuronidase as a reporter protein was fused to the promoter elements of the nisin gene and NisK/NisR were expressed independently in the cell. A dual-plasmid expression system was developed for homologous overproduction in *L. lactis* and heterologous expression in *Leuconostoc lactis* and *Lactobacillus helveticus* (68).

It had been considered for a long time that the genes for nisin were organized in three operons: *NisABTCIP*, *NisRK* and *NisFEG*. However, it would be difficult to explain how the producer organism *L. lactis* can produce sufficient immunity protein NisI to protect itself from being killed before nisin is produced since the genes for NisA and NisI were in the same operon and expression of both requires induction by extracellular nisin. A recent study found the existence of an internal promoter for transcription of NisI and NisP (77), suggesting that the expression of NisI and NisP may not completely depend on induction by the pre-existing nisin so that immunity can establish in the producer before nisin can be produced to bactericidal levels.

The structural gene for subtilin precursor encodes 56 amino acids with 24 residues for the leader peptide and 32 residues for the mature subtilin (4). Although subtilin is produced by *Bacillus subtilis* which is in a different genus, the structure of subtilin is similar to that of nisin. Both peptides contain five intramolecular thio-rings at the same residues. The genetic organization for subtilin is also similar to that for nisin (4, 42, 69-71, 109). Related genes encode proteins that are similar to those for nisin: SpaBTC to NisBTC, SpaIFEG to NisIFEG and SpaRK to NisRK. However, the location of the subtilin structural gene is unique. It is contained in the middle of the gene cluster while the nisin structural gene is the first open reading frame in the cluster. Also, genes *SpaIFEG* encoding immunity proteins for subtilin are clustered in a single operon while *NisI* and *NisFEG* are separated to two operons. The protease encoded by *NisP* which cleaves the nisin precursor is absent in the gene cluster of subtilin. Instead, the subtilin precursor is cleaved by an unspecific serine protease secreted by *B. subtilis*. Similarly, the biosynthesis of subtilin is regulated by quorum-sensing subtilin itself through a histidine kinase/response regulator two-component system encoded by *SpaK* and *SpaR* (67).

Genetic organization and biosynthesis varies among other lantibiotics such as lacticin 481, lacticin 3147, epidermin, pep5, cinnamycin, mersacidin, cytolysin and sapB (128). Production of cytolycin was also found to be auto-regulatory by a two-component regulatory system (54). No lantibiotics other than nisin, subtilin and cytolysin were shown to be the signaling molecules for their own biosynthesis thus far.

Different from class I bacteriocins which are mostly chromosome-associated, the majority of class IIa bacteriocins are plasmid-encoded with the exceptions of enterocin A, divercin V41, sakacin P, carbobacteriocins B2 and BM1 (38). Class IIa bacteriocins are a prominent group due to their *Listeria*-specific antimicrobial activity and their distinctive structural features such as a highly conserved YGNGV motif in the N-terminus of the mature bacteriocins and a double glycine cleavage site in the leader peptides. Usually 2-4 genes are clustered closely in one or multiple operons for biosynthesis. The first fully characterized bacteriocin in this group is pediocin PA-1 (79). Four open reading frames were discovered for biosynthesis of pediocin PA-1. All four genes are clustered in one operon. The first gene *PedA* encodes the pediocin PA-1 precursor, followed by the second gene *PedB* for immunity, the third and forth genes *PedC* and *PedD* for an accessory protein for processing the precursor and for an ATP binding cassette (ABC) transporter, respectively (119). However, biosynthesis of enterocin P only requires two genes, one encoding the enterocin P precursor and the other one encoding the immunity protein. Genes encoding maturation- and transport-related proteins are missing in the locus of enterocin P. This type of bacteriocin relies on the general *sec*-pathway for bacteriocin maturation and transport (28). The leader peptide of enterocin P is highly homologous to *sec*-dependent signal peptides, suggesting that enterocin P is exported by the general *sec*-pathway. Biosynthesis of some bacteriocins requires more than four genes. Fore example, enterocin A (3, 87), sakacins A and P (2, 58) and divercin V41 (80) require additional genes encoding a

three-component regulatory system that controls biosynthesis by quorum-sensing an exported induction peptide. The three component genes which usually are contained in the upstream region of the bacteriocin structural gene, encode an induction peptide, a histidine kinase and a response regulator. The induction peptide acts as a cell-density signal that may activate the kinase by phosphorylation which in turn activates the response regulator by transphosphorylation. The activated response regulator can up-regulate the transcription of the bacteriocin genes including the structural gene. The biosynthesis of class IIa bacteriocins is regulated in a similar manner compared to the class I bacteriocins. The difference is that the production of class IIa bacteriocins is inducible by peptides that are different in sequence from the bacteriocins, while class I bacteriocins such as nisin and subtilin are autoinducers themselves. The induction peptides for class IIa bacteriocins are produced as prepeptides in cells before maturation. It should be noted that only part of class IIa bacteriocins have three-component regulatory system; i.e. most class IIa bacteriocins are produced constitutively. Besides bacteriocins whose production requires 2, 4 or more than 4 genes, the recently identified mundticins indicated that three genes are sufficient for production (8, 65, 100). Three genes for mundticins are clustered in two operons with the structural gene in the first operon and the genes for the ABC transporter and the immunity protein in the second operon. The genetic organization for mundticins is different from other class IIa bacteriocins whose structural genes are usually clustered together with the immunity gene in the same operon.

Two-peptide bacteriocins are classified as class IIb. Both peptides are required for functioning of their antimicrobial activity although either one of the peptides may have low activity. The genetic organization of two-peptide bacteriocins are similar to class IIa bacteriocins: two structural genes encoding two peptide precursors, one gene encoding an immunity protein, two genes encoding an accessory protein whose

function is not well know and an ABC transporter. Therefore, five genes are the minimum for biosynthesis of two-peptide bacteriocins (88). Similarly, production of some members of class IIb bacteriocins is regulated by three-component regulatory system which has been described above for class IIa bacteriocins.

Class IIc consists of circular bacteriocins. The N and C termini of the peptides are connected by a peptide bond. Although only a few bacteriocins in this group have been identified, the genetic organization of those well characterized bacteriocins, is similar to other groups of class II bacteriocins. However, most bacteriocins are chromosome-encoded except for acidocin B and enterocin AS-48 whose genes are located in plasmids (78). Their genes are organized in multiple operons encoding the bacteriocin precursors and proteins for processing, transport, immunity and regulation. Although many bacteriocins in this group are produced constitutively, operons for circularin A contain genes encoding a histidine kinase and a response regulator which may be involved in regulation of biosynthesis.

Class IId consists of non-pediocin-like "miscellaneous" bacteriocins that do not have sequence similarity to other bacteriocins. These bacteriocins have narrower antimicrobial spectrum than other bacteriocins. For example, lactococcin A is only inhibitory against *Lactococcus* spp. (56, 114); the antimicrobial spectrum of divergicin A is similarly narrow (130). Only two genes are required for biosynthesis of divergicin A: one encoding divergicin A precursor and the other one encoding an immunity protein. These two genes are clustered closely in one plasmid-associated operon. The leader peptide of divergicin A is a homologue to *sec*-dependent leaders. Heterologous expression of divergicin A implied that two genes are sufficient for production. Unlike divergicin A, genes for lactococcin A include two additional genes: a gene encoding the ABC transporter-like protein and a downstream gene encoding a protein that might be related to secretion.

Immunity and resistance. Although immunity and resistance to bacteriocins are combined for discussion here, they are fundamentally different concepts in the context of bacteriocins. Immunity refers to the mechanism that the bacteriocin-producing microorganisms use to protect themselves from being killed by their own bacteriocins. Resistance to bacteriocins refers to the development of insensitivity in originally sensitive microorganisms.

Combinational expression of genes for NisI, NisF, NisE and NisG in heterologous and sensitive *Bacillus subtilis* indicated that either NisI or NisFEG confer protection against nisin (107). However, coordinated expression of all four genes provided strongest immunity to nisin, suggesting NisI and NisFEG are playing roles in self-immunity of the nisin producer *L. lactis*. Studies showed that subtilin immunity proteins SpaIFEG function in a manner that is similar to that of nisin (108). Although high similarity in genetics and mechanism exists in nisin- and subtilin-producers, cross-immunity was not observed. Most of other lantibiotic-producers also rely on similar mechanism for self-immunity. How these immunity proteins recognize active lantibiotics and how they interact to protect the producers are not well understood (37). For class IIa bacteriocins, a single immunity protein is responsible for self-immunity. Immunity proteins showed high specificity for their cognitive bacteriocins although a few highly similar proteins provide cross-immunity to multiple bacteriocins (45). It was observed that the specificity of certain immunity proteins depends on which host strain they are expressed, suggesting that strain-specific factors could be involved in immunity. It may explain why some bacteriocin-negative bacteria are naturally insensitive to bacteriocins. This hypothesis was supported by the finding that an immunity protein OrfY which had no known cognitive bacteriocin broadened the immunity to multiple bacteriocins (13, 45). More research is needed to address this poorly understood but fundamentally important area. Little is known about the

molecular action of the immunity protein. It was found that immunity proteins may not interact extensively with the membrane due to their electrostatic polarity of the surface patches and due to the consistency of their three-dimensional structures in both aqueous and membrane-mimicking environments (63). It implied that the cognitive bacteriocin and the immunity protein would be located on the opposite sides of the cytoplasmic membrane, which raised a question of whether any more potential factors in the membrane could be involved in the action of immunity. A recent study shed light on the mysteries of why target bacterial strains are deferentially immune to bacteriocins and how immunity proteins act to protect producers. Diep and co-workers showed evidence for the existence of a membrane receptor for lactococcin A and class IIa bacteriocins (35). The mannose phosphotransferase system (man-PTS) appeared to be a receptor for the antimicrobial action of bacteriocin. Heterologous expression of the man-PTS operon rendered insensitive strains susceptible to lactococcin A and class IIa bacteriocins such as enterocin P, pediocin PA-1, sakacin A and penocin A. The man-PTS system consists of subunits IIAB, IIC and IID. Experimental evidence showed that IIC and IID are the targets for these bacteriocins. In the bacteriocin producer cell, the immunity protein, instead of directly sequestering bacteriocins in the membrane as many researchers had hypothesized, can form a complex with IIC and IID so that an "on-off" type transformation can function to render the producer cell immune to its own bacteriocin. Therefore, man-PTS may serve a universal receptor for many bacteriocins and immunity proteins complex with it to protect the producer cells.

Although development of resistance to bacteriocins in naturally sensitive strains is a rare phenotype compared to bacterial resistance to antibiotics, spontaneous resistance of *L. monocytogenes* to bacteriocins was observed (32, 52, 81). Frequency of spontaneous resistance to nisin and pediocin is in the range of 10^{-2}-10^{-7} depending on the strain and food system. Cross-resistance to multiple bacteriocins was also observed

(81). Stability of resistance varied with different bacteriocins and strains. However, nisin and class IIa bacteriocin resistance comes along with varying degrees of growth inhibition. It was found that the expression of the man-PTS operon was down-regulated or missing in highly resistant mutants of *L. monocytogenes* (53). Alterations in membrane composition of *L. monocytogenes* may also contribute to resistance (31). Questions about how widely bacteriocin resistance can develop in different foods and different species, and how stable resistance can remain are not well answered. No evidence has been established to support cross-resistance between bacteriocins and antibiotics.

Mode of action and antimicrobial spectrum. Nisin can adsorb to target cell by binding to lipid II as a docking molecule and then insert itself into the cytoplasmic membrane to form pores that lead to leakage of solutes of the target cell and cell death (12). By docking lipid II, which is a precursor for peptidoglycan, nisin also inhibits biosynthesis of the cell wall. Several other lantibiotics were shown to act in a similar manner (27). The finding of lipid II as a receptor for nisin suggests that the diverse sensitivity among different bacteria is due to the differential concentration of lipid II in target cell walls. As discussed before, class IIa bacteriocins and class IId bacteriocins which is represented by lactococcin A, use the man-PTS subunits as a receptor to adsorb to target cells (35) and then form membrane pores. However, more research is needed to address the question whether or not the man-PTS serve as a universal docking molecule for all of class II bacteriocins. It is not known if class IIb and class IIc bacteriocins use the same mechanism although they all permeabilize the membranes of target cells.

In general, class I bacteriocins such as nisin have a wider antimicrobial spectrum than class II bacteriocins. However, the antimicrobial activity of class IIa against *L. monocytogenes* is higher than nisin (64). Nisin is active against *L. monocytogenes*,

Bacillus cereus, *Clostridium botulinum*, *Staphylococcus aureus* and many lactic acid bacteria. Gram-negative bacteria are not sensitive to nisin; but nisin can be bactericidal to Gram-negative bacteria when used in combination with chelating agents such as EDTA to disrupt the outer membrane of Gram-negative cells (110). The broader activity spectrum of nisin is an advantage for inhibiting many pathogenic and spoilage bacteria in foods. However, the narrower spectrum of class IIa bacteriocins is not necessarily a disadvantage for application in foods. There is no doubt that the genus *Listeria* is the most sensitive to class IIa bacteriocins. Besides *Listeria* spp., the sensitivity of other genera is not prominent. Class IIa bacteriocins do not have antimicrobial activity against many lactic acid bacteria, which could be advantageous for application in fermentations where multiple cultures are used. In fermentations, the bacteriocinogenic culture may be co-inoculated with other starter cultures that are naturally insensitive to the bacteriocin and the bacteriocin produced *in situ* can inhibit other unwanted microflora, especially *L. monocytogenes*, which is a main safety threat to cheese and meat products. Two-peptide (class IIb) and cyclic (class IIc) bacteriocins have broad spectrums. Enterocin AS-48, a well studied cyclic bacteriocin, can inhibit Gram-positive and even Gram-negative bacteria including *Salmonella* spp. and *E. coli* (1, 51). The spectrum of class IId bacteriocins is very narrow.

Biological control of human pathogens using bacteriocinogenic lactic acid bacteria as bioprotective cultures may be an alternative strategy to improve sprout safety due to their sustainable antimicrobial properties produced during the whole process of sprouting regardless of seed structures where pathogens could be trapped inside.

The objectives of this study were to isolate naturally occurring LAB from sprouts, characterize potentially new bacteriocins produced by isolates, and to investigate the potential of mild heating and bioprotective LAB cultures as multiple hurdles to decontaminate seeds and control the growth of pathogens on sprouts.

REFERENCES

1. **Abriouel, H., E. Valdivia, A. Galvez, and M. Maqueda.** 1998. Response of *Salmonella choleraesuis* LT2 spheroplasts and permeabilized cells to the bacteriocin AS-48. Appl. Environ. Microbiol. **64:**4623-4626.
2. **Axelsson, L. and A. Holck.** 1995. The genes involved in production of and immunity to sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. J. Bacteriol. **177:**2125-2137.
3. **Aymerich, T., H. Holo, L. S. Havarstein, M. Hugas, M. Garriga, and I. F. Nes.** 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl. Environ. Microbiol. **62:**1676-1682.
4. **Banerjee, S. and J. N. Hansen.** 1988. Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. J. Biol. Chem. **263:**9508-9514.
5. **Bari, M. L., J. Sugiyama, and S. Kawamoto.** 2008. Repeated quick hot-and-chilling treatments for the inactivation of *Escherichia coli* O157:H7 in mung bean and radish seeds. Foodborne Pathog. Dis. **6:** 137-143
6. **Bari, M. L., E. Nazuka, Y. Sabina, S. Todoriki, and K. Isshiki.** 2003. Chemical and irradiation treatments for killing *Escherichia coli* O157:H7 on alfalfa, radish, and mung bean seeds. J. Food Prot. **66:**767-774.
7. **Bennik, M. H., W. van Overbeek, E. J. Smid, and L. G. Gorris.** 1999. Biopreservation in modified atmosphere stored mungbean sprouts: the use of vegetable-associated bacteriocinogenic lactic acid bacteria to control the growth of *Listeria monocytogenes*. Lett. Appl. Microbiol. **28:**226-232.

8. **Bennik, M. H., B. Vanloo, R. Brasseur, L. G. Gorris, and E. J. Smid.** 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: full characterization and interaction with target organisms. Biochim. Biophys. Acta. **1373:**47-58.
9. **Beuchat, L. R.** 1997. Comparison of chemical treatments to kill *Salmonella* on alfalfa seeds destined for sprout production. Int. J. Food Microbiol. **34:**329-33.
10. **Beuchat, L. R. and A. J. Scouten.** 2002. Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J. Appl. Microbiol. **92:**382-395.
11. **Beuchat, L. R., T. E. Ward, and C. A. Pettigrew.** 2001. Comparison of chlorine and a prototype produce wash product for effectiveness in killing *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J. Food Prot. **64:**152-158.
12. **Breukink, E., I. Wiedemann, C. van Kraaij, O. P. Kuipers, H. Sahl, and B. de Kruijff.** 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science. **286:**2361-2364.
13. **Brurberg, M. B., I. F. Nes, and V. G. H. Eijsink.** 1997. Pheromone-induced production of antimicrobial peptides in *Lactobacillus*. Mol. Microbiol. **26:**347-360.
14. **Buchman, G. W., S. Banerjee, and J. N. Hansen.** 1988. Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. J. Biol. Chem. **263:**16260-16266.
15. **Cai, Y., L. K. Ng, and J. M. Farber.** 1997. Isolation and characterization of nisin-producing *Lactococcus lactis* subsp. *lactis* from bean sprouts. J. Appl. Microbiol. **83:**499-507.

16. **Centers for Disease Control and Prevention.** 2009. Eat a colorful variety of fruits and vegetables everyday for better health. http://www.fruitsandveggiesmatter.gov.
17. **Centers for Disease Control and Prevention (CDC).** 2009. Outbreak of *Salmonella* serotype Saintpaul infections associated with eating alfalfa sprouts - United States, 2009. MMWR Morb. Mortal. Wkly. Rep. **58:**500-503.
18. **Centers for Disease Control and Prevention (CDC).** 2008. Outbreak of *Salmonella* serotype Saintpaul infections associated with multiple raw produce items - United States, 2008. MMWR Morb. Mortal. Wkly. Rep. **57:**929-934.
19. **Centers for Disease Control and Prevention (CDC).** 2007. Annual listing of foodborne disease outbreaks, United States. http://www.cdc.gov/foodborneoutbreaks/documents/2007/entire_report.pdf.
20. **Centers for Disease Control and Prevention (CDC).** 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach - United States, September 2006. MMWR Morb. Mortal. Wkly. Rep. **55:**1045-1046.
21. **Centers for Disease Control and Prevention (CDC).** 2006. Annual listing of foodborne disease outbreaks, United States. http://www.cdc.gov/foodborneoutbreaks/documents/2006_line_list/2006_line_list.pdf.
22. **Centers for Disease Control and Prevention (CDC).** 2004. Annual listing of foodborne disease outbreaks, United States. http://www.cdc.gov/foodborneoutbreaks/us_outb/fbo2004/Outbreak_Linelist_Final_2004.pdf.

23. **Centers for Disease Control and Prevention (CDC).** 2003. Annual listing of foodborne disease outbreaks, United States. http://www.cdc.gov/foodborneoutbreaks/us_outb/fbo2003/2003LineList.pdf.
24. **Centers for Disease Control and Prevention (CDC).** 2002. Annual listing of foodborne disease outbreaks, United States. http://www.cdc.gov/foodborneoutbreaks/us_outb/fbo2002/2002linelist.pdf.
25. **Centers for Disease Control and Prevention (CDC).** 2001. Annual listing of foodborne disease outbreaks, United States. http://www.cdc.gov/foodborneoutbreaks/us_outb/fbo2001/2001linelists.pdf.
26. **Charkowski, A. O., C. Z. Sarreal, and R. E. Mandrell.** 2001. Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. J. Food Prot. **64:**1292-1298.
27. **Chatterjee, C., M. Paul, L. Xie, and W. A. van der Donk.** 2005. Biosynthesis and mode of action of lantibiotics. Chem. Rev. **105:**633-684.
28. **Cintas, L. M., P. Casaus, L. S. Havarstein, P. E. Hernandez, and I. F. Nes.** 1997. Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. **63:**4321-4330.
29. **Cleveland, J., T. J. Montville, I. F. Nes, and M. L. Chikindas.** 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. **71:**1-20.
30. **Cotter, P. D., C. Hill, and R. P. Ross.** 2005. Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. **3:**777-788.
31. **Crandall, A. D. and T. J. Montville.** 1998. Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Appl. Environ. Microbiol. **64:**231-237.

32. **Davies, E. A. and M. R. Adams.** 1994. Resistance of *Listeria monocytogenes* to the bacteriocin nisin. Int. J. Food Microbiol. **21:**341-347.

33. **de Jong, B., J. Oberg, and B. Svenungsson.** 2007. Outbreak of salmonellosis in a restaurant in Stockholm, Sweden, September-October 2006. Euro Surveill. **12:**E13-4.

34. **deRuyter, P. G. G. A., O. P. Kuipers, and W. M. deVos.** 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. **62:**3662-3667.

35. **Diep, D. B., M. Skaugen, Z. Salehian, H. Holo, and I. F. Nes.** 2007. Common mechanisms of target cell recognition and immunity for class II bacteriocins. Proc. Natl. Acad. Sci. U. S. A. **104:**2384-2389.

36. **Doyle, M. P. and M. C. Erickson.** 2008. Summer meeting 2007 - the problems with fresh produce: an overview. J. Appl. Microbiol. **105:**317-330.

37. **Draper, L. A., R. P. Ross, C. Hill, and P. D. Cotter.** 2008. Lantibiotic immunity. Curr. Protein Pept. Sci. **9:**39-49.

38. **Drider, D., G. Fimland, Y. Hechard, L. M. McMullen, and H. Prevost.** 2006. The continuing story of class IIa bacteriocins. Microbiol. Mol. Biol. Rev. **70:**564-582.

39. **Emberland, K. E., S. Ethelberg, M. Kuusi, L. Vold, L. Jensvoll, B. A. Lindstedt, K. Nygard, C. Kjelso, M. Torpdahl, G. Sorensen, T. Jensen, S. Lukinmaa, T. Niskanen, and G. Kapperud.** 2007. Outbreak of *Salmonella* Weltevreden infections in Norway, Denmark and Finland associated with alfalfa sprouts, July-October 2007. Euro Surveill. **12:**E071129.4.

40. **Engelke, G., Z. Gutowski-Eckel, M. Hammelmann, and K. D. Entian.** 1992. Biosynthesis of the lantibiotic nisin: genomic organization and membrane localization of the NisB protein. Appl. Environ. Microbiol. **58:**3730-3743.

41. **Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki.** 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. **24:**85-106.

42. **Entian, K. D. and W. M. deVos.** 1996. Genetics of subtilin and nisin biosyntheses - Biosynthesis of lantibiotics. Antonie Van Leeuwenhoek. **69:**109-117.

43. **Ferguson, D. D., J. Scheftel, A. Cronquist, K. Smith, A. Woo Ming, E. Anderson, J. Knutsen, A. K. De, and K. Gershman.** 2005. Temporally distinct *Escherichia coli* 0157 outbreaks associated with alfalfa sprouts linked to a common seed source - Colorado and Minnesota, 2003. Epidemiol. Infect. **133:**439-447.

44. **Fett, W. F.** 2006. Inhibition of *Salmonella enterica* by plant-associated pseudomonads in vitro and on sprouting alfalfa seed. J. Food Prot. **69:**719-728.

45. **Fimland, G., V. G. Eijsink, and J. Nissen-Meyer.** 2002. Comparative studies of immunity proteins of pediocin-like bacteriocins. Microbiology. **148:**3661-3670.

46. **Food Standards Agency.** 2004. Risk of food poisoning due to the presence of human pathogens in sprouted seeds. http://www.food.gov.uk/multimedia/pdfs/seedsseminarsummary.pdf,

47. **Franz, E. and A. H. van Bruggen.** 2008. Ecology of *E. coli* O157:H7 and *Salmonella enterica* in the primary vegetable production chain. Crit. Rev. Microbiol. **34:**143-161.

48. **Franz, E., A. V. Semenov, A. J. Termorshuizen, O. J. de Vos, J. G. Bokhorst, and A. H. van Bruggen.** 2008. Manure-amended soil characteristics affecting the survival of *E. coli* O157:H7 in 36 Dutch soils. Environ. Microbiol. **10:**313-327.

49. **Gagliardi, J. V. and J. S. Karns.** 2002. Persistence of *Escherichia coli* O157:H7 in soil and on plant roots. Environ. Microbiol. **4:**89-96.

50. **Galvez, A., H. Abriouel, R. L. Lopez, and N. Ben Omar.** 2007. Bacteriocin-based strategies for food biopreservation. Int. J. Food Microbiol. **120:**51-70.
51. **Galvez, A., E. Valdivia, M. Martinez, and M. Maqueda.** 1989. Bactericidal action of peptide antibiotic AS-48 against *Escherichia coli* K-12. Can. J. Microbiol. **35:**318-321.
52. **Gravesen, A., A. M. J. Axelsen, J. M. da Silva, T. B. Hansen, and S. Knochel.** 2002. Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. Appl. Environ. Microbiol. **68:**756-764.
53. **Gravesen, A., M. Ramnath, K. B. Rechinger, N. Andersen, L. Jansch, Y. Hechard, J. W. Hastings, and S. Knochel.** 2002. High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes*. Microbiology-SGM. **148:**2361-2369.
54. **Haas, W., B. D. Shepard, and M. S. Gilmore.** 2002. Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. Nature. **415:**84-87.
55. **Himathongkham, S., S. Nuanualsuwan, H. Riemann, and D. O. Cliver.** 2001. Reduction of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in artificially contaminated alfalfa seeds and mung beans by fumigation with ammonia. J. Food Prot. **64:**1817-1819.
56. **Holo, H., O. Nilssen, and I. F. Nes.** 1991. Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. J. Bacteriol. **173:**3879-3887.
57. **Hu, H., J. J. Churey, and R. W. Worobo.** 2004. Heat treatments to enhance the safety of mung bean seeds. J. Food Prot. **67:**1257-1260.

58. **Huhne, K., L. Axelsson, A. Holck, and L. Krockel.** 1996. Analysis of the sakacin P gene cluster from *Lactobacillus sake* Lb674 and its expression in sakacin-negative *L. sake* strains. Microbiology-UK. **142:**1437-1448.

59. **Ibekwe, A. M., P. M. Watt, P. J. Shouse, and C. M. Grieve.** 2004. Fate of *Escherichia coli* O157:H7 in irrigation water on soils and plants as validated by culture method and real-time PCR. Can. J. Microbiol. **50:**1007-1014.

60. **Jack, R. W., J. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59:**171-200.

61. **Jaquette, C. B., L. R. Beuchat, and B. E. Mahon.** 1996. Efficacy of chlorine and heat treatment in killing *Salmonella* Stanley inoculated onto alfalfa seeds and growth and survival of the pathogen during sprouting and storage. Appl. Environ. Microbiol. **62:**2212-2215.

62. **Jimenez Diaz, R., J. L. Ruiz Barba, D. P. Cathcart, H. Holo, I. F. Nes, K. H. Sletten, and P. J. Warner.** 1995. Purification and partial amino acid sequence of plantaricin S, a bacteriocin produced by *Lactobacillus plantarum* LPCO10, the activity of which depends on the complementary action of two peptides. Appl. Environ. Microbiol. **61:**4459-4463.

63. **Johnsen, L., G. Fimland, D. Mantzilas, and J. Nissen-Meyer.** 2004. Structure-function analysis of immunity proteins of pediocin-like bacteriocins: C-terminal parts of immunity proteins are involved in specific recognition of cognate bacteriocins. Appl. Environ. Microbiol. **70:**2647-2652.

64. **Katla, T., K. Naterstad, M. Vancanneyt, J. Swings, and L. Axelsson.** 2003. Differences in susceptibility of *Listeria monocytogenes* strains to sakacin P, sakacin A, pediocin PA-1, and nisin. Appl. Environ. Microbiol. **69:**4431-4437.

65. **Kawamoto, S., J. Shima, R. Sato, T. Eguchi, S. Ohmomo, J. Shibato, N. Horikoshi, K. Takeshita, and T. Sameshima.** 2002. Biochemical and genetic

characterization of mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. Appl. Environ. Microbiol. **68:**3830-3840.

66. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39-85.
67. **Kleerebezem, M.** 2004. Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis. Peptides **25:**1405-1414.
68. **Kleerebezem, M., M. M. Beerthuyzen, E. E. Vaughan, W. M. deVos, and O. P. Kuipers.** 1997. Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. Appl. Environ. Microbiol. **63:**4581-4584.
69. **Klein, C. and K. D. Entian.** 1994. Genes involved in self-protection against the lantibiotic subtilin produced by *Bacillus subtilis* ATCC 6633. Appl. Environ. Microbiol. **60:**2793-2801.
70. **Klein, C., C. Kaletta, and K. D. Entian.** 1993. Biosynthesis of the lantibiotic subtilin is regulated by a histidine kinase response regulator system. Appl. Environ. Microbiol. **59:**296-303.
71. **Klein, C., C. Kaletta, N. Schnell, and K. D. Entian.** 1992. Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl. Environ. Microbiol. **58:**132-142.
72. **Kostrzynska, M. and A. Bachand.** 2006. Use of microbial antagonism to reduce pathogen levels on produce and meat products: a review. Can. J. Microbiol. **52:**1017-1026.
73. **Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. Devos.** 1993. Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis* - requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur. J. Biochem. **216:**281-291.

74. **Kuipers, O. P., M. M. Beerthuyzen, P. G. G. A. Deruyter, E. J. Luesink, and W. M. Devos.** 1995. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal-transduction. J. Biol. Chem. **270:**27299-27304.

75. **Kulasekara, B. R., M. Jacobs, Y. Zhou, Z. Wu, E. Sims, C. Saenphimmachak, L. Rohmer, J. M. Ritchie, M. Radey, M. McKevitt, T. L. Freeman, H. Hayden, E. Haugen, W. Gillett, C. Fong, J. Chang, V. Beskhlebnaya, M. K. Waldor, M. Samadpour, T. S. Whittam, R. Kaul, M. Brittnacher, and S. I. Miller.** 2009. Analysis of the genome of the *Escherichia coli* O157:H7 2006 spinach-associated outbreak isolate indicates candidate genes that may enhance virulence. Infect. Immun. **77:** 3713-3721.

76. **Kurzer, M. S. and X. Xu.** 1997. Dietary phytoestrogens. Annu. Rev. Nutr. **17:**353-381.

77. **Li, H. and D. J. OSullivan.** 2006. Identification of a *nisI* promoter within the *nisABCTIP* operon that may enable establishment of nisin immunity prior to induction of the operon via signal transduction. J. Bacteriol. **188:**8496-8503.

78. **Maqueda, M., M. Sanchez Hidalgo, M. Fernandez, M. Montalban-Lopez, E. Valdivia, and M. Martinez-Bueno.** 2008. Genetic features of circular bacteriocins produced by Gram-positive bacteria. FEMS Microbiol. Rev. **32:**2-22.

79. **Marugg, J. D., C. F. Gonzalez, B. S. Kunka, A. M. Ledeboer, M. J. Pucci, M. Y. Toonen, S. A. Walker, L. C. Zoetmulder, and P. A. Vandenbergh.** 1992. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, and bacteriocin from *Pediococcus acidilactici* PAC1.0. Appl. Environ. Microbiol. **58:**2360-2367.

80. **Metivier, A., M. F. Pilet, X. Dousset, O. Sorokine, P. Anglade, M. Zagorec, J. C. Piard, D. Marion, Y. Cenatiempo, and C. Fremaux.** 1998. Divercin V41, a new bacteriocin with two disulphide bonds produced by *Carnobacterium*

divergens V41: primary structure and genomic organization. Microbiology-UK **144:**2837-2844.

81. **Naghmouchi, K., E. Kheadr, C. Lacroix, and I. Fliss.** 2007. Class I/class IIa bacteriocin cross-resistance phenomenon in *Listeria monocytogenes*. Food Microbiol. **24:**718-727.
82. **National Advisory Committee on Microbiological Criteria for Foods.** 1999. Microbiological safety evaluations and recommendations on sprouted seeds. Int. J. Food Microbiol. **52:**123-153.
83. **Neetoo, H., T. Pizzolato, and H. Chen.** 2009. Elimination of *Escherichia coli* O157:H7 from alfalfa seeds through a combination of high hydrostatic pressure and mild heat. Appl. Environ. Microbiol. **75:**1901-1907.
84. **Neetoo, H., M. Ye, and H. Chen.** 2009. Factors affecting the efficacy of pressure inactivation of *Escherichia coli* O157:H7 on alfalfa seeds and seed viability. Int. J. Food Microbiol. **131:**218-223.
85. **Neetoo, H., M. Ye, and H. Chen.** 2008. Potential application of high hydrostatic pressure to eliminate *Escherichia coli* O157:H7 on alfalfa sprouted seeds. Int. J. Food Microbiol. **128:**348-353.
86. **Nes, I. F., D. B. Diep, L. S. Havarstein, M. B. Brurberg, V. Eijsink, and H. Holo.** 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek. **70:**113-128.
87. **OKeeffe, T., C. Hill, and R. P. Ross.** 1999. Characterization and heterologous expression of the genes encoding enterocin a production, immunity, and regulation in *Enterococcus faecium* DPC1146. Appl. Environ. Microbiol. **65:**1506-1515.

88. **Oppegard, C., P. Rogne, L. Emanuelsen, P. E. Kristiansen, G. Fimland, and J. Nissen-Meyer.** 2007. The two-peptide class II bacteriocins: structure, production, and mode of action. J. Mol. Microbiol. Biotechnol. **13:**210-219.
89. **Palmai, M. and R. L. Buchanan.** 2002. Growth of *Listeria monocytogenes* during germination of alfalfa sprouts. Food Microbiol. **19:**195-200.
90. **Park, C. M., P. J. Taormina, and L. R. Beuchat.** 2000. Efficacy of allyl isothiocyanate in killing enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. Int. J. Food Microbiol. **56:**13-20.
91. **Pfeiler, E. A. and T. R. Klaenhammer.** 2007. The genomics of lactic acid bacteria. Trends Microbiol. **15:**546-553.
92. **Pierre, P. M. and E. T. Ryser.** 2006. Inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104, and *Listeria monocytogenes* on inoculated alfalfa seeds with a fatty acid-based sanitizer. J. Food Prot. **69:**582-590.
93. **Pineiro, M. and C. Stanton.** 2007. Probiotic bacteria: legislative framework - requirements to evidence basis. J. Nutr. **137:**850S-3S.
94. **Portnoy, B. L., J. M. Goepfert, and S. M. Harmon.** 1976. An outbreak of *Bacillus cereus* food poisoning resulting from contaminated vegetable sprouts. Am. J. Epidemiol. **103:**589-594.
95. **Proctor, M. E., M. Hamacher, M. L. Tortorello, J. R. Archer, and J. P. Davis.** 2001. Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa sprouts grown from seeds pretreated with calcium hypochlorite. J. Clin. Microbiol. **39:**3461-3465.
96. **Public Health Agency of Canada.** 2006. Outbreak of *Salmonella* Enteritidis phage type 13 associated with mung bean sprouts in Ontario, 2005. http://www.sproutnet.com/Research/Toronto%20%20Mung%202005.pdf.

97. **Rajkowski, K. T. and D. W. Thayer.** 2001. Alfalfa seed germination and yield ratio and alfalfa sprout microbial keeping quality following irradiation of seeds and sprouts. J. Food Prot. **64:**1988-1995.

98. **Rajkowski, K. T. and D. W. Thayer.** 2000. Reduction of *Salmonella* spp. and strains of *Escherichia coli* O157:H7 by gamma radiation of inoculated sprouts. J. Food Prot. **63:**871-875.

99. **Rajkowski, K. T., G. Boyd, and D. W. Thayer.** 2003. Irradiation D-values for *Escherichia coli* O157:H7 and *Salmonella* spp. on inoculated broccoli seeds and effects of irradiation on broccoli sprout keeping quality and seed viability. J. Food Prot. **66:**760-766.

100. **Saavedra, L., C. Minahk, A. P. de Ruiz Holgado, and F. Sesma.** 2004. Enhancement of the enterocin CRL35 activity by a synthetic peptide derived from the NH_2-terminal sequence. Antimicrob. Agents Chemother. **48:**2778-2781.

101. **Salminen, S. and A. von Wright.** 1993. Lactic acid bacteria. Marcel Dekker, Inc., New York.

102. **Saroj, S. D., S. Hajare, R. Shashidhar, V. Dhokane, A. Sharma, and J. R. Bandekar.** 2007. Radiation processing for elimination of *Salmonella* Typhimurium from inoculated seeds used for sprout making in India and effect of irradiation on germination of seeds. J. Food Prot. **70:**1961-1965.

103. **Scouten, A. J. and L. R. Beuchat.** 2002. Combined effects of chemical, heat and ultrasound treatments to kill *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J. Appl. Microbiol. **92:**668-674.

104. **Semenov, A. V., L. van Overbeek, and A. H. van Bruggen.** 2009. Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. Appl. Environ. Microbiol. **75:**3206-3215.

105.Settanni, L. and A. Corsetti. 2008. Application of bacteriocins in vegetable food biopreservation. Int. J. Food Microbiol. **121:**123-138.

106.Sivapalasingam, S., C. R. Friedman, L. Cohen, and R. V. Tauxe. 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. J. Food Prot. **67:**2342-2353.

107.Stein, T., S. Heinzmann, I. Solovieva, and K. D. Entian. 2003. Function of *Lactococcus lactis* nisin immunity genes *nisI* and *nisFEG* after coordinated expression in the surrogate host *Bacillus subtilis*. J. Biol. Chem. **278:**89-94.

108.Stein, T., S. Heinzmann, S. Dusterhus, S. Borchert, and K. D. Entian. 2005. Expression and functional analysis of the subtilin immunity genes *spaIFEG* in the subtilin-sensitive host *Bacillus subtilis* MO1099. J. Bacteriol. **187:**822-828.

109.Stein, T., S. Borchert, P. Kiesau, S. Heinzmann, S. Kloss, C. Klein, M. Helfrich, and K. D. Entian. 2002. Dual control of subtilin biosynthesis and immunity in Bacillus subtilis. Mol. Microbiol. **44:**403-416.

110.Stevens, K. A., B. W. Sheldon, N. A. Klapes, and T. R. Klaenhammer. 1991. Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. Appl. Environ. Microbiol. **57:**3613-3615.

111.Stewart, D. S., K. F. Reineke, J. M. Ulaszek, and M. L. Tortorello. 2001. Growth of *Salmonella* during sprouting of alfalfa seeds associated with salmonellosis outbreaks. J. Food Prot. **64:**618-622.

112.Stiles, M. E. 1996. Biopreservation by lactic acid bacteria. Antonie Van Leeuwenhoek. **70:**331-345.

113.Stiles, M. E. and W. H. Holzapfel. 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. **36:**1-29.

114.Stoddard, G. W., J. P. Petzel, M. J. van Belkum, J. Kok, and L. L. McKay. 1992. Molecular analyses of the lactococcin A gene cluster from *Lactococcus*

lactis subsp. *lactis* biovar diacetylactis WM4. Appl. Environ. Microbiol. **58:**1952-1961.

115.Taormina, P. J. and L. R. Beuchat. 1999. Comparison of chemical treatments to eliminate enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. J. Food Prot. **62:**318-324.

116.Thayer, D. W., K. T. Rajkowski, G. Boyd, P. H. Cooke, and D. S. Soroka. 2003. Inactivation of *Escherichia coli* O157:H7 and *Salmonella* by gamma irradiation of alfalfa seed intended for production of food sprouts. J. Food Prot. **66:**175-181.

117.Tyler, H. L. and E. W. Triplett. 2008. Plants as a habitat for beneficial and/or human pathogenic bacteria. Annu. Rev. Phytopathol. **46:**53-73.

118.U.S. Food and Drug Administration. 1999. Consumers advised of risks associated with raw sprouts. http://vm.cfsan.fda.gov/~lrd/hhssprts.html.

119.Venema, K., J. Kok, J. D. Marugg, M. Y. Toonen, A. M. Ledeboer, G. Venema, and M. L. Chikindas. 1995. Functional analysis of the pediocin operon of *Pediococcus acidilactici* Pac1.0: pedB is the immunity protein and pedD is the pPrecursor processing enzyme. Mol. Microbiol. **17:**515-522.

120.Vojdani, J. D., L. R. Beuchat, and R. V. Tauxe. 2008. Juice-associated outbreaks of human illness in the United States, 1995 through 2005. J. Food Prot. **71:**356-364.

121.Weiss, A. and W. P. Hammes. 2005. Efficacy of heat treatment in the reduction of salmonellae and *Escherichia coli* O157 : H- on alfalfa, mung bean and radish seeds used for sprout production. Eur. Food Res. Technol. **221:**187-191.

122.Weiss, A. and W. P. Hammes. 2003. Thermal seed treatment to improve the food safety status of sprouts. J. Appl. Bot./Angewandte Botanik. **77:**152-155.

123.Weissinger, W. R. and L. R. Beuchat. 2000. Comparison of aqueous chemical treatments to eliminate *Salmonella* on alfalfa seeds. J. Food Prot. **63:**1475-1482.

124.Weissinger, W. R., K. H. McWatters, and L. R. Beuchat. 2001. Evaluation of volatile chemical treatments for lethality to *Salmonella* on alfalfa seeds and sprouts. J. Food Prot. **64:**442-450.

125.Werner, S., K. Boman, I. Einemo, M. Erntell, B. de Jong, A. Lindqvist, M. Lofdahl, S. Lofdahl, A. Meeuwisse, G. Ohlen, M. Olsson, U. Stamer, E. Sellstrom, and Y. Andersson. 2007. Outbreak of *Salmonella* Stanley in Sweden associated with alfalfa sprouts, July-August 2007. Euro Surveill. **12:**E071018.2.

126.Western Australia Department of Health. 2006. Hundreds may be affected by *Salmonella* outbreak. http://www.health.wa.gov.au/press/view_press.cfm?id=561.

127.Wilderdyke, M. R., D. A. Smith, and M. M. Brashears. 2004. Isolation, identification, and selection of lactic acid bacteria from alfalfa sprouts for competitive inhibition of foodborne pathogens. J. Food Prot. **67:**947-951.

128.Willey, J. M. and W. A. van der Donk. 2007. Lantibiotics: peptides of diverse structure and function. Annu. Rev. Microbiol. **61:**477-501.

129.Wood, B. J. B. and P. J. Warner. 2003. Genetics of lactic acid bacteria. Kluwer Academic/Plenum Publishers, New York.

130.Worobo, R. W., M. J. Van Belkum, M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles. 1995. A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. J. Bacteriol. **177:**3143-3149.

CHAPTER 2

THERMAL INACTIVATION OF *Salmonella* spp. AND *Escherichia coli* O157:H7 ON ALFALFA SEEDS[1]

ABSTRACT

Alfalfa seeds inoculated with five strains of *Salmonella* spp. or *E. coli* O157:H7 were subjected to dry heat at 55ºC for up to 8 days. Five-log reductions in *Salmonella* spp. or *E. coli* O157:H7 on seeds were observed. No pathogens were detected on the sprouted seeds which were initially inoculated with ca. 2 log CFU/g of *Salmonella* spp. or more than 8 log CFU/g of *E. coli* O157:H7. The germination percentages of the alfalfa seeds did not significantly decrease after 6 days of heating at 55ºC. These results showed that heat treatment of alfalfa seeds at 55ºC for up to 6 days was effective in enhancing the safety of alfalfa sprouts without affecting germination significantly.

INTRODUCTION

Since the first large international outbreak of salmonellosis in the United States and Finland in 1995, the safety of eating fresh raw sprouts, especially alfalfa sprouts has been a major concern for consumers (21). The pathogens found on seeds are thought to originate from environmental sources during seed production at the crop level. The contamination levels of seeds are usually low in numbers (21). However, during sprouting, temperatures of approximately 30ºC and high humidity create favorable conditions for the rapid growth of pathogens that initially may be at below detectable

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levels on seeds but increase to more than 10^7 CFU/g within 24 hours of sprouting, which pose a health risk to consumers.

A number of physical and chemical methods have been evaluated for their effectiveness in inhibiting or eliminating bacterial foodborne pathogens on seeds and sprouts. Soaking alfalfa seeds inoculated with 2.4-3.23 log CFU/g of *E. coli* O157:H7 in 20,000 ppm active chlorine, in combination with heating at 55 ºC was found to be effective in reducing pathogen levels to below detectable levels. However, pathogens were still detected by enrichment (20). In response to this finding, the U.S. Food and Drug Administration (FDA) recommended that soaking alfalfa seeds in 20,000 ppm calcium hypochlorite for 15 min, be used as a seed decontamination treatment prior to sprouting. However, in 1999 still another multi-state outbreak of salmonellosis due to alfalfa sprouts occurred despite the sprout manufacturer following FDA recommended chlorine treatment prior to sprouting. This suggested that the hypochlorite treatment of the seeds insufficiently reduced the levels of contaminating pathogens associated with the seeds used for sprouting (17). Although it was reported that 20,000 ppm free chlorine soak for 10 min was still effective in eliminating *Salmonella* Mbandaka from naturally contaminated seeds (19), other studies showed that the efficacy of 20,000 ppm chlorine was questionable in eliminating *Salmonella* spp., which grew to more than 7 log CFU/g on sprouts even with spray application of 100 ppm chlorine during germination (10). Re-growth of *E. coli* O157:H7 to 7 log CFU/g on sprouts after 20,000 ppm hypochlorite treatment for 15 min has also been observed (14). Many other studies have examined the effectiveness of various chemicals including chlorine at various concentrations (3-5, 11, 13, 15, 16, 18, 24, 25), indicating that none of the sanitizers were able to eradicate *Salmonella* or *E. coli* O157:H7 without affecting germination, although significant reduction of the pathogens was observed. Physical treatments, including Gamma irradiation (22) and heating in hot water (23) as a means

to decontaminate *Salmonella* and *E. coli* were confirmed to be capable of reducing the population on seeds and sprouts significantly, but complete elimination was not observed. High hydrostatic pressure can reduce *E. coli* O157:H7 populations by 2 logs but results in extremely poor germination of the seeds (1). Biocontrol of *Salmonella* spp. by plant-associated pseudomonads was studied and found to be effective in inhibiting the growth of *Salmonella* but was unable to reduce the populations on sprouts (9).

It is the physiology of alfalfa seeds that complicates the disinfection of pathogenic bacteria. Researchers have postulated that removing pathogens from alfalfa seeds is more difficult than from other seeds because alfalfa seeds have wrinkles (7) and crevices on the surface, allowing pathogens inside these protective structures and making them inaccessible to chemical sanitizers. The biofilms formed by pathogens on sprouts may provide protection against antimicrobial compounds used for decontamination (8).

Heat treatment, by soaking seeds in hot water, has been tried by many researchers, resulting in limited reduction of the pathogens and usually decreases in seed germination rates (18, 19, 23). Dry heat has been shown to be an effective decontamination treatment for mung bean without affecting the seed viability (12). However, dry heat has not been extensively studied for alfalfa seeds. Dry heat could be a promising treatment, because heat transfer will not be hindered by seed physiological structures, such as crevices or wrinkles that provide protection to pathogens when chemical treatments are applied to alfalfa seeds.

The purpose of this study was to investigate the effects of dry heat on the viability of *E. coli* O157:H7 and *Salmonella*, the two most frequent pathogens that have been responsible for the widespread outbreaks associated with alfalfa sprout consumption, and the effect on the viability of alfalfa seeds with various heat treatments.

MATERIALS AND METHODS

Cultures and Media. Five serovars of *Salmonella enterica* and five different strains of *E. coli* O157:H7, were used as inocula: *Salmonella* Gaminara, *Salmonella* Hartford, *Salmonella* Typhimurium, *Salmonella* Rubislaw and *Salmonella* Montevideo; *E. coli* O157:H7 ATCC 43889 (clinical isolate), ATCC 43894 (clinical isolate), ATCC 43895 (raw hamburger meat isolate), 933 (raw hamburger meat isolate), provided by M. P. Doyle, Center for Food Safety, University of Georgia, and ATCC 35150 (clinical isolate) provided by J. Russell, Department of Microbiology, Cornell University. To facilitate the enumeration of colonies on tryptic soy agar (TSA), all of the strains were transformed by electroporation with the plasmid encoding for green fluorescence protein (pGFP) and ampicillin antibiotic resistance.

Inoculation and treatment. For high inoculation levels on alfalfa seeds, each serovar of *S. enterica* or each strain of *E. coli* O157:H7 was loop-inoculated into 50 ml tryptic soy broth (TSB; Criterion, Hardy Diagnostics, CA, USA) containing ampicillin at 100 mg/L (Fisher Scientific, NJ, USA). Each 50 ml TSB with inoculum was incubated at 37 ºC with 250-rpm shaking for 18 h (Innova 4230, New Brunswick Scientific, Edison, NJ, USA). After incubation, five 50-ml strains of *E. coli* O157:H7 or five serovars of *S. enterica* were mixed to make a cocktail of 250 ml inoculum. The cocktail was combined with 200 ml sterile deionized distilled water (Milli-Q RG, Millipore, Bedford, MA, USA) and 60 g of alfalfa seeds of the Lucerne variety (Springwater Sprouts Inc., Honeoye Falls, NY). Seeds were soaked in a 500 ml sterile beaker for 20 min at 20ºC with occasional agitation.

For low inoculation levels, each serovar of *S. enterica* or each strain of *E. coli* O157:H7 was loop-inoculated into 5 ml of TSB containing ampicillin (100 mg/L). The cultures were incubated as described above. After incubation, every culture of *E. coli* O157:H7 was serially diluted in TSB 1,000-fold and *Salmonella* by 10,000-fold. One

milliliter of each diluted strain was combined to create a mix of all five strains or serovars for making a 5-ml cocktail of *S. enterica* or *E. coli* O157:H7. Sixty grams of seeds were soaked in 1 liter of sterile deionized distilled water inoculated with 5 ml of *S. enterica* or *E. coli* O157:H7 cocktails for 20 min at 20ºC with occasional agitation.

After soaking, the seeds were poured onto two layers of sterile cheesecloth, which was laid on a raised stainless steel mesh (10 by 25 cm; Hoeltge Inc., Cincinnati, OH). Seeds were evenly spread to form a uniform thickness of approximately 5 mm and allowed to dry overnight in a biological safety cabinet (Nuaire Inc., Plymouth, MN).

Eight grams of dried inoculated seeds were placed into 15-ml sterile plastic centrifuge tubes (Fisherbrand, Fisher) and heated at 55 ºC for 4, 6 and 8 days in the same incubator described above. The capped tubes were placed horizontally on a rack to allow the seeds to spread evenly over the tube interior, ensuring equal heating.

Germination. After 0, 4, 6 and 8 days of incubation at 55 ºC, 1 g of seeds (ca. 550 seeds) were placed into 50 ml sterile centrifuge tubes (Simport Plastics, Beloeil, Quebec, Canada). The tubes were filled with sterile distilled water to 50 ml. Seeds were soaked for 4 h with occasional agitation. The seeds were poured onto a 3M filter paper (Fisher) in a sterile plastic container (12 by 8 by 6 cm) that was used to simulate the normal sprouting conditions. The filter paper was placed on top of a sponge inside the plastic container to provide humidity during germination. The seeds were evenly spread over the filter paper with a sterile glass rod to ensure every seed was in contact with the filter paper.

The seeds were incubated at 30 ºC for 3 days before the germination rate of the seeds and pathogen population levels of the sprouts were determined. During the time of germination, sterile distilled water was sprayed on the seeds/sprouts twice daily (12 h apart) to maintain the amount of moisture the seeds require for sprouting. The germination percentage was calculated by dividing the number of germinated seeds by

the total number of seeds in the sample at the time of germination. The treatments and germinations were performed in triplicate in separate containers. Ruptured seeds with cotyledons still intact inside the cuticle were not considered germinated but enumerated as part of the total seed numbers.

Bacterial Analysis. After inoculation, drying or heating, 1 gram of the alfalfa seeds was sampled and mixed with 9 ml of 0.1 % peptone water in a 60-ml sterile stomacher bag (Fisherbrand, Fisher). The bag was stomached at 260 rpm for 60 s (Stomacher 400, Seward Ltd., Basingstoke, UK). The homogenate was serially diluted in sterile 0.1% peptone water. For the seeds inoculated with *Salmonella*, duplicate 0.1-ml of each dilution and quadruplicate 0.25-ml portions of the undiluted mixture were surface plated onto TSA (Difco, Becton, Dickinson, Sparks, MD) supplemented with ampicillin (100 mg/L) and onto Hektoen enteric Agar (HEA; Criterion, Hardy); for the seeds inoculated with *E. coli* O157:H7, the dilutions were surface plated by the same method as described above except for the use of violet red bile agar with MUG (4-methylumbellifery-β-glucuronide) (VRBA; Difco, Becton, Dickinson) in lieu of HEA.

When the seeds had been sprouted for 3 days, 3-6 g of the sprouts were combined 10-fold with 0.1 % peptone water. Three separate containers were sampled and assayed for surviving populations of *Salmonella* and *E. coli* O157:H7 on sprouts by the same method mentioned above.

TSA plus ampicillin medium and VRBA were incubated at 37 ºC for 24 h, and HEA was incubated for 48 h. The enumeration of the colonies on TSA plus ampicillin medium was done under UV light of 320 nm. Only the colonies that exhibited green fluorescence were counted on TSA plus ampicillin. Typical colonies on HEA and VRBA were also counted. Suspected colonies that appeared to be *E. coli* O157:H7 or *Salmonella* spp. were confirmed using Chromagar O157 and Chromagar Salmonella (BBL, Becton Dickinson, Sparks, MD).

All the experiments were performed in separate triplicate trials with duplicate plating.

Statistical Methods. The percentages of germination from a series of treatment times were compared with the control (no heating time) to determine if heat treatments reduce the germination significantly by the Dunnett method. Analysis of variance between treatments with different heating times was used to compare mean values of pathogen population on sprouts. All of the statistical analyses were conducted by the software JMP 5.1.2 (SAS Institute Inc., Cary, NJ).

RESULTS

Remaining *Salmonella* populations. As Table 2.1 indicates, the levels of *Salmonella* on seeds decreased compared with the untreated control seeds. However, *Salmonella* on sprouts from all treatments and control seeds rapidly increased to approximately the same levels during sprouting, regardless of the treatment and *Salmonella* reduction achieved on the seeds. The increase in *Salmonella* levels on the sprouts is even much higher considering the increase in weight and surface area that occurs during the sprouting process.

It is noticeable that overnight drying of freshly inoculated seeds resulted in approximately 1-log reduction of *Salmonella*. Similar results were also found in the subsequent experiments of this study. With the high inoculation level of 8.00 log CFU/g, *Salmonella* was inactivated by more than 5 logs on seeds after 6 days of heating at 55ºC (Table 2.1). After 8 days of heating, the population of *Salmonella* on seeds was below detection limits (1 log CFU/g or 10 CFU/g of seeds). After 3 days of sprouting with favorable conditions for pathogen growth (incubation at 30 ºC and water spraying), the populations of the pathogens from different batches of sprouts treated for 4 d, 6 d and 8 d were not significantly different from each other (P>0.05, not marked in Table 2.1), suggesting that the final pathogen populations on the sprouts

were not related to the different treatment times as long as there was *Salmonella* surviving on or inside the seeds. On TSA plus ampicillin, pathogen populations on the seeds were below the detection limit after 8 d of heating, while it was already undetectable on HEA, even after 6 d of heating. However, after 3 d of sprouting, the pathogens grew quickly to high numbers on sprouts even though it was below detectable limit on the seeds. Although the numbers of *Salmonella* on sprouts were lower than that of the control, it is obvious that with high inoculation levels, heating at 55ºC had little effect on the reduction of the pathogen on sprouts, while it was easy to obtain a 5-log reduction on seeds. The likely reason for this observation is that there were surviving pathogens on seeds, although they were too few to be detected, and they could grow to high numbers on sprouts during the sprouting process, which allows rapid pathogen growth. It was found that, normally, there was a difference in pathogen counts between TSA plus ampicillin and HEA, especially for sprouts. The difference could be attributed to the lack of recovery for heat-injured cells and a proportion of the total cells that lost the pGFP plasmid and, as a consequence, were no longer ampicillin resistant or capable of producing green fluorescent protein. However, plasmidless cells grew on the selective medium HEA, resulting in an enumeration difference.

After the seed inoculation level was lowered to ~2 log CFU/g, the pathogen was still capable of growing to high numbers on sprouts after the sprouting process from untreated seeds (control) (Table 2.1), although on TSA plus ampicillin, it was below detection limits prior to sprouting. As the results of both TSA plus ampicillin and HEA show, the pathogens on all heat treated seeds for 4 d, 6 d and 8 d were not detected. Furthermore, no pathogens were detected on sprouts after 3 d of sprouting at 30ºC, indicating that heating process is effective in eradicating the pathogens from the seeds.

Table 2.1. Thermal inactivation (at 55ºC for up to 8 days) of *Salmonella* on alfalfa seeds at high and low inoculum levels of 8.00 (8.69) log CFU/g and 2.17(2.63) log CFU/g, respectively.

Heating time (days)	*Salmonella* (Log CFU/g seeds or sprouts) on seeds and sprouts with high inoculum level		*Salmonella* (Log CFU/g seeds or sprouts) on seeds and sprouts with low inoculum level	
	After heating	After 3d of sprouting	After heating	After 3d of sprouting
0	NA (NA)	6.64±0.14 (ND)	NA (NA)	4.35±1.50 (7.04±0.09)
4	2.14±0.14 (1.49±0.20)	5.92±0.14 (8.50±0.12)	<1 (<1)	<1 (<1)
6	1.32±0.28 (<1)	5.82±0.91 (8.61±0.36)	<1 (<1)	<1 (<1)
8	<1 (<1)	5.73±0.45 (8.91±0.09)	<1 (<1)	<1 (<1)

Note: After drying, the levels of *Salmonella* on seeds were 6.79 (7.19) log CFU/g and <1(1) log CFU/g for high and low inoculum levels, respectively. Data were from TSA plus ampicillin and from HEA (in parentheses). Values are means of triplicates except those under detection limit. NA, not applicable. ND, not determined.

Remaining *E. coli* O157:H7 populations. *E. coli* O157:H7 remained on the sprouts at almost the same level of cell concentrations on the seeds that did not undergo heat treatment (Table 2.2). For the seeds with high inoculation levels, the pathogens on the seeds were detected at low numbers on TSA plus ampicillin while on VRBA no cells were detected on the seeds after 4 d of heating at 55ºC. The reduction of more than 5 log on seeds was achieved by heating the seeds at 55ºC for 4 days or more. However, during sprouting, *E. coli* O157:H7 quickly grew to high levels even if it was below the detection limits on VRBA from the seeds that underwent 4 days of heating. After 6 days of treatment, the pathogens were below detectable limits on the seeds.

Table 2.2. Thermal inactivation (at 55ºC for up to 8 days) of *E. coli* O157:H7 on alfalfa seeds at high and low inoculum levels of 8.75 (8.68) log CFU/g and 3.15(3.00) log CFU/g, respectively.

Heating time (days)	*E. coli* O157:H7 (Log CFU/g seeds or sprouts) on seeds and sprouts with high inoculum level		*E. coli* O157:H7 (Log CFU/g seeds or sprouts) on seeds and sprouts with low inoculum level	
	After heating	After 3d of sprouting	After heating	After 3d of sprouting
0	NA (NA)	8.59±0.08 (8.59±0.10)	NA (NA)	7.43±0.22 (7.44±0.13)
4	1.6 (<1)	6.96±0.27 (6.88±0.23)	<1 (<1)	5.73 (6.17±0.44)
6	<1 (<1)	<1 (<1)	<1 (<1)	<1 (<1)
8	<1 (<1)	<1 (<1)	<1 (<1)	<1 (<1)

Note: After drying, the levels of *E. coli* O157:H7 on seeds were 7.29(6.82) log CFU/g and 2.33 (2.02) log CFU/g for high and low inoculum levels, respectively. Data were from TSA plus ampicillin and from VRBA (in parentheses). Values are means of triplicates except those under detection limit. NA, not applicable.

Table 2.3. The germination percentages of alfalfa seeds heated at 55ºC for up to 8 days with (A) and without inoculation procedures (B).

Heating time at 55ºC	Germination Percentages (%)	
	Treatment A	Treatment B
Positive Control (0d)	82.0±3.5 a	82.8±0.7 a
4d	62.0±6.5 b	78.7±4.0 a
6d	59.7±4.2 b	76.8±2.9 a
8d	47.0±3.7 b	74.6±2.0 b

Note: All data are mean values of separate triplicates. Values in the same column followed by a letter that is different from that of the control are significantly different from the control (P<0.05). Treatment A was inoculation of the alfalfa seeds with high level of *Salmonella*, followed by drying the seeds in a laminar flow hood and then heating the seeds at 55 ºC for up to 8 days; Treatment B was heating the seeds in the same method but without an inoculation procedure, imitating the industrial procedure.

After sprouting, *E. coli* O157:H7 was still below detectable limits on the sprouts. Therefore, the temperature and time regime of this heat treatment was effective in killing high levels of *E. coli* O157:H7 on sprouts.

The effect of heat treatment on low levels of inoculation was also investigated. Seeds were inoculated with *E. coli* O157:H7 cocktail at the level of ~3 log CFU/g (Table 2.2). A ca. 1-log reduction was also observed after the seeds were dried in the flow hood. The pathogens on untreated seeds grew to more than 7 log CFU/g on sprouts after the sprouting process. Heating the seeds at 55ºC for 4 days was effective in reducing pathogen levels to below detectable limits. However, large numbers of *E. coli* O157:H7 were still observed after sprouting. For 6 days or more of heating, no pathogens were observed on both TSA plus ampicillin and VRBA media. Furthermore, pathogens were under detection limits on sprouts, suggesting that the treatment of 55°C for 6 days had eradicated the pathogens from the seeds.

Germination percentages of treated seeds. After inoculation with high levels of *Salmonella*, drying in the laminar flow biological safety cabinet, heating at 55 ºC for up to 8 days and sprouting the seeds for 3 days, the germination percentages were obtained by counting the sprouts from the different treatments. It was found that the germination of the seeds decreased significantly (P<0.05) from 82.0% to 62.0%, 59.7% and 47.0% for 4, 6 and 8 days of heating at 55°C, respectively, when compared with the control (Table 2.3).

The germination of the seeds that underwent heat treatment without inoculation procedures was investigated to determine the extra effects, if any, of the inoculation on seed viability. The percentage of germination of the heat treated seeds still remained at 78.7% and 76.8% with 4 and 6 days of heating, respectively. The percentages of germination of the seeds that were heated for 4 and 6 days were not significantly different from the control, 82.8% (P>0.05). After 8 days of heating, the germination

decreased to 74.60%, which was significantly different ($P<0.05$) from the control. The overall appearance of the treated sprouts was not different from the control.

DISCUSSION

From the results obtained in this study, it was possible to achieve a 5-log reduction of both *Salmonella* and *E. coli* O157:H7 on alfalfa seeds without significantly affecting the seed viability. However, after the sprouting process, both *Salmonella* and *E. coli* O157:H7 were shown to grow to dangerously high levels of 5 to 7 log CFU/g or more than 7 log CFU/g, even though they were not detected on the seeds after the heat treatment (Tables 2.1 and 2.2). This suggests that pathogen enumeration of the sprouts is crucial, even after seed decontamination treatments.

Seeds inoculated at high levels of *E. coli* O157:H7 and heat treated at 55°C for ≥4 days resulted in alfalfa sprouts that were below detectable levels. However, only seeds inoculated with low inoculum levels of *Salmonella* and heat treated at 55°C for ≥4 days resulted in sprouts that were below detectable levels. These results suggest that the higher heat resistance of the *Salmonella* strains allowed for lower inactivation rates in seeds and consequently the presence of *Salmonella* in sprouts.

It has been reported that *Salmonella* grows faster (6) and attaches more tightly to the surfaces of the alfalfa sprouts (2) than does *E. coli* O157:H7 during the sprouting process. In this case, *Salmonella* might recover more quickly from heat stress than *E. coli* O157:H7. Therefore, *Salmonella* is more recalcitrant for the elimination from alfalfa seeds.

It was observed that with alfalfa sprouts, there was a large gap in the numbers of typical colonies of *Salmonella* formed on TSA plus ampicillin and HEA media, while the numbers of *E. coli* O157:H7 on TSA plus ampicillin were in close agreement with those on VRBA. This observed difference might be attributable to the instability of the

plasmid (pGFP) in *Salmonella* cells. Current studies are being performed to confirm this hypothesis.

In this study, heating alfalfa seeds at 55ºC for 4 or more days was capable of achieving a >5 log reduction in both *Salmonella* and *E. coli* O157:H7 populations, which greatly enhanced the safety of alfalfa sprouts. This method is effective only in removing low levels of *Salmonella* contamination on seeds, while it is effective in eradicating high contamination levels of *E. coli* O157:H7 at more than 8-log CFU/g contamination levels. Under normal conditions, the pathogens contaminate the seeds at very low numbers before harvest. The effectiveness of this method to decontaminate pathogens at artificially high pathogen levels should be sufficient for naturally contaminated seeds at much lower pathogen contamination levels. Higher temperatures were attempted and showed faster kill rates, but the germination rates were significantly reduced. The germination of alfalfa seeds was greatly reduced at temperatures higher than 55 ºC after several days of holding (data not shown).

The difference in the percentages of germination of heated seeds with and without inoculation could be attributed to the inoculation effect on the viability of the seeds. To be inoculated, the seeds were soaked in the inoculum for 20 min and then dried overnight in the laminar flow biological safety cabinet. These procedures could add to the effects of heat in reducing the germination rate of the alfalfa seeds. The combined effects of inoculation and heating resulted in the significant reduction of seed viability. The results from the heated seeds without inoculation indicated that heat treatment at 55ºC for up to 6 days did not reduce the percentage of germination significantly, and even after 8 days of heating, the germination was still acceptable. The percentages of germination obtained in this study showed that dry heat at 55ºC could be an effective treatment for ensuring the safety of the alfalfa sprouts without affecting the viability of the alfalfa seeds significantly.

Dry heat is more practical and promising, because the germination of alfalfa seeds appeared to be less sensitive to dry heat than to heat in hot water. Hot-water treatment for alfalfa seeds at around 55ºC or more than 55ºC was previously reported to be detrimental to seed viability, even for several minutes of treatment (13, 18, 23). However, this study indicates that the germination of alfalfa seeds was stable for several days of dry heat at 55ºC, suggesting that dry heat may be applicable to the sprout industry both in terms of safety assurance and of maintaining an acceptable percentage of germination for the producers.

It is the wrinkled surface seed structure of alfalfa seeds that makes the eradication of pathogens from seeds difficult. Some pathogens remain inside the seed coat, wrinkled grooves or crevices that are not accessible to chemical sanitizers; hence, there is incomplete access to the contaminating pathogens by chemical sanitizers that largely work as surface sanitizers. However, heat transfer can penetrate the food matrix, regardless of the intricate structure of the seeds. Therefore, heat treatment can be an effective treatment for removing even internalized or protected pathogens from alfalfa seeds.

The results of this study showed that heat treatment of seeds at 55ºC for up to 6 days can be an effective method for ensuring the safety of the alfalfa sprouts without reducing the germination of the seeds significantly. Heat treatment may be a promising alternative to current ineffective methods because the treatment can easily be applied to large quantities of seeds prior to selling to sprout producers. In addition, the application is easily monitored and recordable to ensure the proper treatment was applied.

REFERENCES

1. **Ariefdjohan, M. W., P. E. Nelson, R. K. Singh, A. K. Bhunia, V. M. Balasubramaniam, and N. Singh.** 2004. Efficacy of high hydrostatic pressure treatment in reducing *Escherichia coli* O157 and *Listeria monocytogenes* in alfalfa seeds. J. Food Sci. **69:** M117-M120.
2. **Barak, J. D., L. C. Whitehand, and A. O. Charkowski.** 2002. Differences in attachment of *Salmonella enterica* serovars and *Escherichia coli* O157:H7 to alfalfa sprouts. Appl. Environ. Microbiol. **68:** 4758-4763.
3. **Beuchat, L. R.** 1997. Comparison of chemical treatments to kill *Salmonella* on alfalfa seeds destined for sprout production. Int. J. Food Microbiol. **34:** 329-333.
4. **Beuchat, L. R., and A. J. Scouten.** 2002. Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J. Appl. Microbiol. **92:** 382-395.
5. **Beuchat, L. R., T. E. Ward, and C. A. Pettigrew.** 2001. Comparison of chlorine and a prototype produce wash product for effectiveness in killing *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J. Food Prot. **64:** 152-158.
6. **Charkowski, A. O., J. D. Barak, C. Z. Sarreal, and R. E. Mandrell.** 2002. Differences in growth of *Salmonella enterica* and *Escherichia coli* O157:H7 on alfalfa sprouts. Appl. Environ. Microbiol. **68:** 3114-3120.
7. **Charkowski, A. O., C. Z. Sarreal, and R. E. Mandrell.** 2001. Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. J. Food Prot. **64:** 1292-1298.
8. **Fett, W. F.** 2000. Naturally occurring biofilms on alfalfa and other types of sprouts. J. Food Prot. **63:** 625-632.
9. **Fett, W. F.** 2006. Inhibition of Salmonella enterica by plant-associated pseudomonads in vitro and on sprouting alfalfa seed. J. Food Prot. **69:** 719-728.

10. **Gandhi, M., and K. R. Matthews.** 2003. Efficacy of chlorine and calcinated calcium treatment of alfalfa seeds and sprouts to eliminate *Salmonella*. Int. J. Food Microbiol. **87:** 301-306.
11. **Himathongkham, S., S. Nuanualsuwan, H. Riemann, and D. O. Cliver.** 2001. Reduction of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in artificially contaminated alfalfa seeds and mung beans by fumigation with ammonia. J. Food Prot. **64:** 1817-1819.
12. **Hu, H., J. J. Churey, and R. W. Worobo.** 2004. Heat treatments to enhance the safety of mung bean seeds. J. Food Prot. **67:** 1257-1260.
13. **Jaquette, C. B., L. R. Beuchat, and B. E. Mahon.** 1996. Efficacy of chlorine and heat treatment in killing *Salmonella* Stanley inoculated onto alfalfa seeds and growth and survival of the pathogen during sprouting and storage. Appl. Environ. Microbiol. **62:** 2212-2215.
14. **Lang, M. M., B. H. Ingham, and S. C. Ingham.** 2000. Efficacy of novel organic acid and hypochlorite treatments for eliminating *Escherichia coli* O157:H7 from alfalfa seeds prior to sprouting. Int. J. Food Microbiol. **58:** 73-82.
15. **Park, C. M., P. J. Taormina, and L. R. Beuchat.** 2000. Efficacy of allyl isothiocyanate in killing enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. Int. J. Food Microbiol. **56:** 13-20.
16. **Pierre, P. M., and E. T. Ryser.** 2006. Inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104, and Listeria monocytogenes on inoculated alfalfa seeds with a fatty acid-based sanitizer. J. Food Prot. **69:** 582-590.
17. **Proctor, M., M. Hamacher, M. Tortorello, J. Archer, and J. Davis.** 2001. Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa sprouts grown from seeds pretreated with calcium hypochlorite. J. Clin. Microbiol. **39:** 3461-3465.

18. **Scouten, A. J., and L. R. Beuchat.** 2002. Combined effects of chemical, heat and ultrasound treatments to kill *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J. Appl. Microbiol. **92:** 668-674.
19. **Suslow, T. V., J. Wu, W. F. Fett, and L. J. Harris.** 2002. Detection and elimination of *Salmonella* Mbandaka from naturally contaminated alfalfa seed by treatment with heat or calcium hypochlorite. J. Food Prot. **65:** 452-458.
20. **Taormina, P., and L. Beuchat.** 1999. Comparison of chemical treatments to eliminate enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. J. Food Prot. **62:** 318-324.
21. **Taormina, P., L. Beuchat, and L. Slutsker.** 1999. Infections associated with eating seed sprouts: an international concern. Emerg. Infect. Dis. **5:** 626-633.
22. **Thayer, D. W., K. T. Rajkowski, G. Boyd, P. H. Cooke, and D. S. Soroka.** 2003. Inactivation of *Escherichia coli* O157:H7 and *Salmonella* by gamma irradiation of alfalfa seed intended for production of food sprouts. J. Food Prot. **66:** 175-181.
23. **Weiss, A., and W. P. Hammes.** 2005. Efficacy of heat treatment in the reduction of salmonellae and *Escherichia coli* O157:H$^-$ on alfalfa, mung bean and radish seeds used for sprout production. Eur. Food Res. Technol. **221:** 187-191.
24. **Weissinger, W. R., K. H. McWatters, and L. R. Beuchat.** 2001. Evaluation of volatile chemical treatments for lethality to *Salmonella* on alfalfal seeds and sprouts. J. Food Prot. **64:** 442-450.
25. **Weissinger, W. R., and L. R. Beuchat.** 2000. Comparison of aqueous chemical treatments to eliminate *Salmonella* on alfalfa seeds. J. Food Prot. **63:** 1475-1482.

CHAPTER 3

CHARACTERIZATION OF MUNDTICIN L, A CLASS IIA ANTI-*Listeria* BACTERIOCIN FROM *Enterococcus mundtii* CUGF08[2]

ABSTRACT

***Enterococcus mundtii* CUGF08, a lactic acid bacterium isolated from alfalfa sprouts, was found to produce a bacteriocin. The bacteriocin, designated mundticin L, has high inhibitory activity specifically against the genus *Listeria*, including the foodborne pathogen *Listeria monocytogenes* and several other genera. Hybridization studies revealed that the gene cluster for mundticin L is contained on a large plasmid. The genetic determinants required for the bacteriocin production, including the structural gene for mundticin L, the genes for the ATP binding cassette (ABC) transporter and the immunity protein were cloned and sequenced. The precursor of mundticin L is comprised of 58 amino acids and a double glycine processing site at the C-terminus of the leader peptide. Mundticin L consists of 43 amino acids with an intramolecular disulfide bond following a YGNGX motif in the N-terminus, which is typical of class IIa bacteriocins. Compared to the homologous mundticin KS or ATO6 and enterocin CRL35, mundticin L has a unique leucine residue instead of the most commonly found valine as the fifth residue in the YGNGX motif. The amino acid sequence of the leader peptide is identical to that of CRL35 but different at residue positions –6 (Ala) and –10 (Ser) from that of mundticin KS. The sequences of the ABC transporter and immunity protein contains 674 and 98 amino acids,**

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respectively, and differ from those reported for KS and CRL35. The gene for mundticin L precursor is located in a single operon while the ABC transporter and the immunity protein are clustered in a separate downstream operon.

INTRODUCTION

Bacteriocins are ribosomally synthesized proteinaceous compounds that inhibit closely related bacteria (22). Due to consumer concerns with chemical and irradiation preservation methods, and the rising demand for minimally processed food products, alternative methods for shelf life extension and enhanced safety are needed. Bacteriocins are considered as "natural" antimicrobials since many bacteriocins are produced by food-grade lactic acid bacteria, which are generally recognized as safe (GRAS).

Bacteriocins can be divided into three main classes: the class I lanthionine-containing lantibiotics, exemplified by nisin; the class II non-lanthionine-containing bacteriocins; and the class III of heat-labile, large proteins (7). Class III bacteriocins have limited application due to their thermal instability and cytolytic activity against eukaryotic cells. Class II can be further divided into class IIa containing pediocin-like bacteriocins, class IIb containing two-peptide bacteriocins, and class IIc of other bacteriocins (10). Class IIa bacteriocins have been extensively studied since pediocin PA-1 was first discovered (15) and characterized (24). Currently, only nisin in class I has been approved by the FDA as a natural food additive. Bacteriocins belonging to class IIa are promising alternative antimicrobials since they are stable over a broader range of heating regiments and pH conditions. In addition, these bacteriocins exhibit stronger antimicrobial activity against the genus *Listeria* than nisin (20) but have a narrower antimicrobial spectrum.

The potential applications of class IIa bacteriocins in both meat and plant-based foods as a means to provide protection against potential foodborne pathogens and

extend shelf life continue to expand. In an attempt to use biological methods for controlling foodborne pathogens on fresh sprouts, a number of food grade lactic acid bacteria were isolated from the indigenous microbiota on alfalfa sprouts. Some of these isolates were found to be bacteriocinogenic. This study describes a new class IIa bacteriocin, mundticin L produced by *Enterococcus mundtii* CUGF08 isolated from alfalfa sprouts.

MATERIALS AND METHODS

Media and Cultures. The mundticin L producer *E. mundtii* CUGF08 was obtained from alfalfa sprouts. MRS broth (Difco, Becton Dickinson and Company, Sparks, MD and Criterion, Santa Maria, CA) was used to grow lactic acid bacteria including the bacteriocin producers *Enterococcus mundtii* CUGF08 and *Enterococcus mundtii* ATO6 (3). The indicator strain *Listeria ivanovii* and other bacteria used for antimicrobial spectrum determination and activity titration were grown in tryptic soy broth (TSB; Difco; Table 3.1). Agar media contained 1.5% (wt/vol) agar (Difco) and soft agar used for overlay contained 0.75% agar. *Escherichia coli* DH5α with the cloning vector pUC19 were grown in LB media (Difco) containing 100 μg/mL of ampicillin (Fisher Scientific, Fair Lawn, NJ). *E. coli* ElectroMAX DH5α-E electrocompetent cells (Invitrogen, Carlsbad, CA) were used for transformation of recombinant pUC19. LB agar containing ampicillin (100 μg/mL), 120 μg/mL of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fisher Scientific) and 40 μg/mL of X-gal (Fisher Scientific) was used to screen transformed cells. Stock cultures were maintained at −80°C in the respective media without antibiotics but supplemented with 15% glycerol (Fisher Scientific).

Identification of the isolates. The genus and species of the isolates were determined by phenotypic and genetic characterization. The 16S rRNA gene was amplified in a 50 μL PCR reaction containing 50 pmol of each primer, 16S-F (5'–

AGAGTTTGATCCTGGCTCAG –3') and 16S-R (5'–AAGGAGGTGATCCAGCCGCA–3') (12), approximately 0.05 µg of chromosomal DNA, 0.2 mM of each deoxynucleoside triphosphates (Fisher Scientific), and 2.5 units of Taq polymerase (Fisher Scientific). PCR was carried out under the following conditions: one step of denaturing at 94°C for 3 min, 30 cycles of denaturing at 94°C for 1 min, annealing at 37°C for 2 min and extension at 72°C for 1.5 min, and one final step of extension at 72°C for 8 min in a RoboCycler (Stratagene, La Jolla, CA). The PCR product was purified by QIAquick gel extraction kit (Qiagen, Germantown, MD) and then sequenced on an Applied Biosystems Automated 3730 DNA Analyzer (Foster City, CA) at Cornell University Life Sciences Core Laboratories Center. The sequence was analyzed for homologous sequences using the BLAST program of National Center for Biotechnology Information (26) to determine the identity of the isolate.

Characterization of bacteriocin. Bacteriocin production was confirmed by performing a deferred inhibition assay as described by Schillinger and Lücke (32), with the following modifications. A pure culture of each isolate was spotted onto MRS agar and incubated at 30°C for 24 h. Proteolytic enzymes were used to confirm the proteinaceous nature of bacteriocins. Ten-microliter aliquots of different proteolytic enzymes, including proteinase K (20mg/ml, Fisher Scientific), pronase E (10 mg/ml, Sigma, Saint Louis, MO) were added close to the 24-h colonies. After drying, 8 ml of soft agar (0.75%, wt/vol) medium containing 0.05 ml of an 18h culture of an indicator strain was overlaid. To eliminate the antimicrobial effect of acids produced by lactic acid bacteria, 10 µl of Tris buffer (2M Tris-HCl, pH 8.0) was added as described above for proteolytic enzymes. The plates were incubated for an additional 24 h. Clear inhibition zones, inactivated by any of the proteolytic enzymes but unaffected by the Tris buffer, were considered as an indication of bacteriocin production by the isolates.

The bacteriocin was extracted (as described below) and subjected to Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) (30, 31) using a Mini-Protean II electrophoresis system (Bio-Rad, Hercules, CA). Duplicate gels were run at 120 V for 1.5 h before they were stained with Coomassie Blue G-250 (Bio-Rad). Ultra-low-range molecular weight marker (molecular weights, 1,060 - 26,600; Sigma) was used to estimate the molecular weight of the unknown bacteriocin. Following fixation with methanol (30%, wt/vol), one of the duplicate gels was overlaid with 8 ml of soft agar containing 50 µL of the late-log-phase sensitive indicator strain.

Determination of antimicrobial activity and spectrum of activity. The activity of the bacteriocin was titrated by a spot diffusion assay (9). The cell-free supernatant containing maximum activity of the bacteriocin was prepared by centrifuging and filtering through a 0.2 µm pore-sized cellulose acetate membrane (VWR International, West Chester, PA). The filtrate was subjected to 2 fold serial dilutions in 50 mM sodium phosphate buffer (pH 7.0) and 10 µL of each dilution was spotted onto a lawn of the indicator strains. The lawns were prepared by overlaying 8 mL of TSB or MRS soft agar inoculated with 50 µL of *Listeria* spp. and other strains over a base agar medium (TSA or MRS; Table 3.1). Following drying in a laminar flow hood, the spotted plates were incubated for 24 hours at 30˚C for lactic acid bacteria and 37˚C for other strains. The antimicrobial activity of the bacteriocin was measured using *Listeria ivanovii* as the indicator strain. The antimicrobial activity was defined as the reciprocal of the highest dilution showing definite inhibition in the indicator lawn. The activity was expressed in arbitrary unit per ml (AU/ml). The antimicrobial spectrum of the bacteriocin and the minimum inhibitory concentration of the tested strains were determined using the same method.

Isolation, cloning and sequencing of the bacteriocin genetic determinants. Standard molecular biology methods described by Sambrook and Russell (29) were used, unless otherwise noted. *E. mundtii* CUGF08 chromosomal DNA was purified from a 30-ml overnight culture using a method described by Mengaud et al. (25), with modifications. The cloning vector pUC19 and subsequent recombinant plasmids from *E. coli* DH5α were purified using a plasmid mini kit (Qiagen) according to the procedures described by the manufacturer. Plasmids from lactic acid bacteria were isolated following the method described by Anderson and McKay (1). PCR products were purified with a QIAquick gel extraction kit. The DNA concentration was determined with a NanoDrop ND 1000 (Thermo Scientific, Waltham, MA). Restriction enzymes were obtained from Fisher Scientific or Promega, Madison, WI.

To isolate the bacteriocin gene cluster, restriction enzyme (Fisher Scientific, and Promega, Madison, WI) digestions of genomic DNA were carried out in 50 µL reactions containing 1-2 µg of DNA for 1-2 h at 37°C. The digested DNA was subjected to electrophoresis at 2 V/cm for 4.5 h before staining with ethidium bromide (0.5 µg/mL). DNA in the gel was alkaline transferred onto a positively charged Hybond-N+ nylon membrane (Amersham, United Kingdom). The DNA was fixed to the membrane at 120 mJ/cm^2 by a UV cross-linker and hybridized with the PCR product from the amplified relevant bacteriocin genes as probes. The probes were labeled with thermostable alkaline phosphatase and detected by GE Amersham AlkPhos Direct Labeling and CDP Star Detection Systems. Developer and fixer solutions and chemiluminescence film (Kodak, Rochester, NY) were used to visualize the hybridization patterns.

The restriction fragments that hybridized with the PCR amplified bacteriocin genes were cloned into the multiple cloning sites of pUC19. Briefly, fragments were recovered from agarose gel after electrophoresis of digested genomic DNA as

described above. DNA in the recovered gel was electroeluted into a dialysis bag (MWCO 500 Da; Spectrum, Rancho Dominguez, CA) at 5 V/cm for 30 min. The electroeluted DNA was purified by chloroform/phenol extraction and standard ethanol precipitation. The cloning vector pUC19 was digested with compatible restriction enzymes that were used to digest the genomic DNA. The vector DNA digested by a single restriction enzyme was dephosphorylated using shrimp alkaline phosphatase (Boehringer Mannheim, Germany) prior to ligation. T4 ligase (Invitrogen) was used to ligate the recovered insert and vector at the molar ratio of 3:1 (9 fmol to 3 fmol) in a 20 μL reaction. The reaction was diluted 5 fold prior to transformation. Transformation was carried out by electroporating 1 μL of the diluted ligation mixture with 20 μL of competent *E. coli* DH5α cells and electroporated at 2.0 kV, 200 Ω and 25 μF (Gene Pulser II, Bio-Rad) in 2.0 mm-gapped electroporation cuvettes (Bio-Rad). The transformed cells were plated to LB agar containing ampicillin, IPTG and X-gal. Colonies from the transformation were blotted and transferred onto nylon membranes (Amersham). The membrane was hybridized by the same method as described above using the PCR amplified product of the bacteriocin gene of interest as probes.

The recombinant plasmids containing the correct inserts were sequenced from both directions using a series of designed primers based on the known nucleotide sequence from the flanking vector and from the insert.

Determination of the genetic locus. The chromosomal and plasmid DNA from the bacteriocin producer was used to determine the genetic locus of the gene cluster responsible for bacteriocin production. Isolated DNA was subjected to electrophoresis in a 0.6% agarose gel. The DNA was blotted onto the nylon membrane and hybridized with the 16S rRNA gene, which was a PCR product using the primers and conditions described above and with a structural gene for the bacteriocin in order to differentiate

between chromosomal and plasmid DNA and to determine the fragment containing the genetic locus.

Nucleotide and amino acid sequence analysis. The cloned nucleotide sequence was analyzed by Vector NTI Advance 10 (Invitrogen). The promoters for operons were determined by a prokaryotic promoter prediction program from Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Netherlands (37). The primary and secondary structure of the bacteriocin was analyzed by the ExPASy Proteomics System (33).

Adsorption/desorption extraction of bacteriocin from producer cells. The bacteriocin was extracted by pH-dependent adsorption and desorption of the bacteriocin on the producer cells (40). The producer strain was grown in 1 liter of MRS at 30°C for 18 h until maximum bacteriocin concentration in the medium was observed. The pH of the culture was adjusted to 6.5 with 1M NaOH. The culture was heated at 70°C for 30 min to kill the producer cells. The cells were collected by centrifugation at 10,000 × *g* for 15 mins at 4°C and the supernatant was removed. The cells were washed twice with 5 mM sodium phosphate (pH 6.5) to remove the medium components. The bacteriocin was released from the cells by resuspending the cells in 50 mL of 100 mM NaCl, pH 2.0, which was adjusted by 5% phosphoric acid. The solution was agitated at 4°C for 1 h. The cells were removed by centrifugation and the supernatant was sterilized by a 0.2 μm-sized cellulose acetate membrane and dialyzed in dialysis tubing (MWCO 2,000 Da, Spectrum) in 3×2 liters of Milli-Q water for 24 h at 4°C with stirring. The resultant solution was lyophilized overnight and reconstituted in 3 ml of 50 mM phosphate buffer (pH 7.0).

Electroblotting and N-terminal sequencing of the bacteriocin. The extracted bacteriocin was subjected to Tricine-SDS-PAGE as described above. The bacteriocin in polyacrylamide gel was electroblotted onto a polyvinylidene fluoride membrane

(Micron Separations Inc., Westborough, MA) in transfer buffer containing 10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% methanol (11) at 100 V for 1 h at 4°C. The blotted bacteriocin band on the membrane was visualized by staining with 0.1% Coomassie Blue R-250 (Bio-Rad), 1% acetic acid, 50% methanol, and destaining in 50% methanol. The bacteriocin band was excised from the membrane and loaded onto a Perkin-Elmer sequencer (Waltham, MA) for Edman degradation (Synthesis and Sequencing Facility, Johns Hopkins University School of Medicine).

RESULTS

Identification and characterization of the bacteriocin and its producer. The putative bacteriocin producer was identified to be *Enterococcus mundtii*, designated as *Enterococcus mundtii* CUGF08, based on sequence homology of the 16S rRNA gene. On MRS medium, *E. mundtii* CUGF08 produced a typical yellow pigment that is distinctive from those of other *Enterococcus* spp (6). It is a homofermentative lactic acid bacterium of plant-associated sources. The putative bacteriocin produced by *E. mundtii* CUGF08 was inactivated by both of pronase E and proteinase K and was not affected by Tris buffer, indicating that the putative antimicrobial compound produced by *E. mundtii* CUGF08 was proteinaceous. Tricine-SDS-PAGE analysis of the extracted bacteriocin and overlay of the Tricine-SDS-PAGE gel with the indicator strain *L. ivanovii*, showed that the molecular weight was approximately 4,000. The bacteriocin was designated mundticin L.

Antimicrobial spectrum. *L. ivanovii* was used as an indicator strain to titer the antimicrobial activity of the bacteriocin preparations since it was the most sensitive strain from the range of indicator bacteria strains tested. *Listeria* spp. were shown to be the most sensitive group of bacteria to mundticin L (Table 3.1), while *E. faecalis* FSL-23 140, a strain that is more related to the producer in taxonomy, was sensitive but much less than the *Listeria* spp. tested.

Table 3.1. Minimum inhibitory concentration of bacterial strains to mundticin L (AU/mL).

Bacterial indicators	Mundticin L (AU/mL)	Incubation conditions for tested strains
Listeria innocua	3200	TSA, 37˚C
Listeria monocytogenes 10403S	400	TSA, 37˚C
Listeria seeligeri	800	TSA, 37˚C
Bacillus subtilis 1A650	256000	TSA, 37˚C
Bacillus cereus ATCC 11778	–	TSA, 37˚C
Bacillus circulans 1473 sm	–	TSA, 37˚C
Staphylococcus aureus ATCC 25923	–	TSA, 37˚C
Pseudomonas fluorescens	–	TSA, 37˚C
Enterococcus faecalis FSL 23-140	6400	MRS, 30˚C
Lactobacillus casei	–	MRS, 30˚C
Lactobacillus fermentum ATCC 14931	–	MRS, 30˚C
Lactococcus lactis ATCC 11454	–	MRS, 30˚C
E. coli O157:H7 ATCC 43895	–	TSA, 37˚C
Salmonella enterica serovar Enteritidis	–	TSA, 37˚C

However, of the various *Listeria* spp. tested, different species showed varied sensitivity to mundticin L, with *L. innocua* being the least sensitive and *L. monocytogenes* 10403S being the most sensitive. Gram negative strains including *Pseudomonas fluorescens*, *E. coli* O157:H7 ATCC 43895 and *Salmonella enteritidis* were not sensitive to mundticin L. Except for *Enterococcus faecalis* FSL 23-140, mundticin L was not active against any of the lactic acid bacteria tested which included *L. casei*, *L. fermentum* ATCC 14931 and the nisin A producer *L. lactis* spp.

lactis 11454. Although not potent, mundticin L showed some inhibitory activity against *Bacillus subtilis* 1A650. *Bacillus cereus* ATCC 11778 however, was not sensitive to mundticin L.

Cloning and sequencing of the genetic determinants. Based on the acquired characteristics including producer identity, molecular size of the bacteriocin and its antimicrobial spectrum, a PCR was performed to detect the mundticin KS structural gene using primers Mnt-1F (5'–TGAGAGAAGGTTTAAGTTTTGAAGAA–3') and Mnt-1R (5'–TCCACTGAAATCCATGAATGA–3') and conditions described by Zendo et al (41). The PCR product showed a 700 bp fragment for the mundticin structural gene using *E. mundtii* CUGF08 as the template. The PCR product was recovered from gel and sequenced. The deduced amino acid sequence from the PCR product was determined to be different from that of mundticin KS.

The genetic determinants for the bacteriocin structural and relevant genes were isolated by Southern blot and hybridization. A 5.5 kb *Kpn*I and a 7.3 kb *Hin*dIII fragments, which contain the mundticin L structural gene, were isolated by hybridizing with the mundticin structural gene from the PCR described above. An additional 1.8 kb *Hin*dIII fragment containing the immunity gene was isolated by hybridizing with a probe which was the immunity gene from a PCR using primers IM-F (5' –GACAAGTGTGACATAATCATTG–3') and IM-R (5'–CAGCATTTTTAAAGATACCAAC–3'), which were designed based on the reported sequence of mundticin KS gene cluster (21). The PCR conditions for the immunity gene were the same to those identified for the mundticin KS gene except for the annealing temperature of 37°C. All three fragments were ligated into pUC19 and cloned in *E. coli* DH5α. The fragments were sequenced from both directions of the flanking DNA of pUC19 using designed primers U19F (5'–GCCTCTTCGCTATTACGCC–3') and U19R (5'–

GTTAGCTCACTCATTAGGCACC–3'), and other walking primers (data not shown) based on the acquired nucleotide sequence of the inserts.

Three open reading frames (ORFs) of the relevant genetic determinants were identified (Figure 3.1). The first ORF (ORF1) encodes the mundticin L prepeptide, which consists of 58 amino acids (Figure 3.1). Following ORF1 is a putative *rho*-independent terminator. ORF2 encodes a putative ATP binding cassette (ABC) transporter with a separate set of transcriptional elements. ORF2 is followed closely by ORF3 encoding for the putative immunity protein for mundticin L. ORF2 and ORF3 are in the same operon, sharing a common transcriptional start site and a *rho*-independent terminator. No additional sequence was found to be associated with the production of mundticin L.

The mature peptide of mundticin L has 43 amino acids, and two cysteine residues in the carboxyl terminus forming a putative disulfide bond (Figure 3.2). The calculated molecular weight for mundticin L is 4301.8, and the pI was 9.45. Mundticin L has a precursor cleavage site after the double glycine residues (Figure 3.2) and the highly conserved YGNGX motif in the N-terminus, both of which are typical of class IIa bacteriocins. However, in the YGNGX motif of mundticin L, the fifth residue was determined to be leucine instead of valine, the most common residue in this motif in class IIa bacteriocins.

Therefore, mundticin L is a new bacteriocin with a single amino acid difference in the YGNGX motif compared to mundticin KS and enterocin CRL35 (27). The electrostatic distribution along the molecule is highly polarized with most of the cationic residues concentrated in the N-terminal region. The N-terminal residues at positions 7 to 9 (Leu-Ser-Cys) and 15 to 17 (Ser-Val-Asp) are predicted (5) to form β strands, followed by two α helices at positions 18 to 25 (Trp-Gly-Lys-Ala-Ile-Gly-Ile-Ile) and 29 to 32 (Ser-Ala-Ala-Asn).

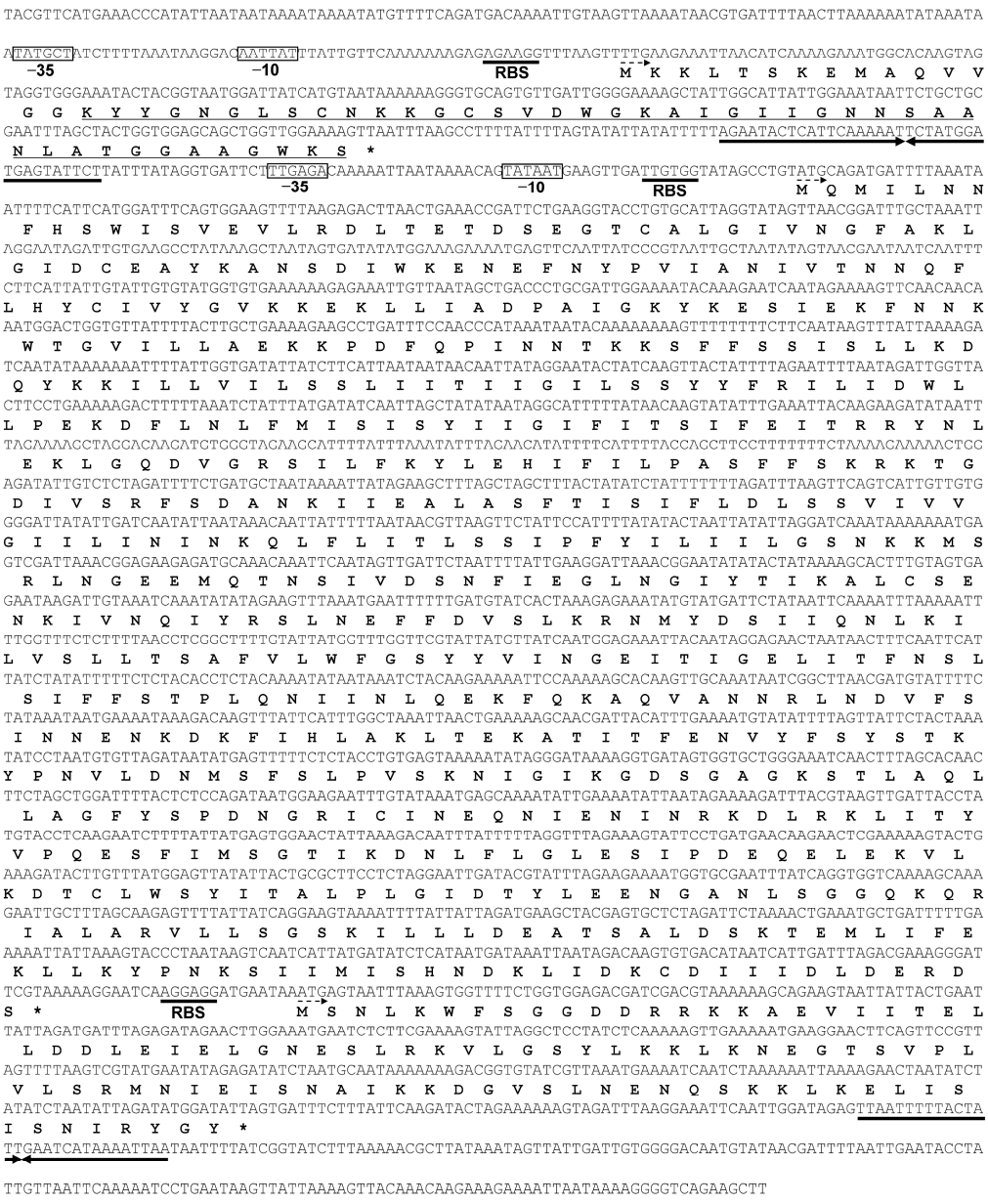

Figure 3.1. The operons for mundticin L production, transport, and immunity. The first ORF encodes the bacteriocin precursor followed by the second ORF for the putative ABC transporter and the third ORF for the putative immunity protein. Amino acid residues in single letters are given under their nucleotide sequence with the start codon underlined by a dotted arrow. The underlined AA sequence is the mature mundticin L. Boxed nucleotides are putative promoters. RBS: ribosome binding site. Sequence underlined by opposite arrows is a putative *rho*-independent terminator.

Besides the single amino acid difference from mundticin KS and enterocin CRL35 in the mature peptide, the leader peptide of mundticin L has alanine and serine residues at positions −6 and −10, respectively, which are identical to the residues of enterocin CRL35 but different from the residues of mundticin KS, which are serine and alanine, respectively. Although the mature peptides of mundticin KS and CRL35 are the same, the sequences of the leader peptides are different for the two bacteriocins (Figure 3.2). Therefore, based on amino acid sequences of the bacteriocin precursors, there are three different bacteriocins in the mundticin group of class IIa bacteriocins: mundticin L, mundticin KS (ATO6) and enterocin CRL35.

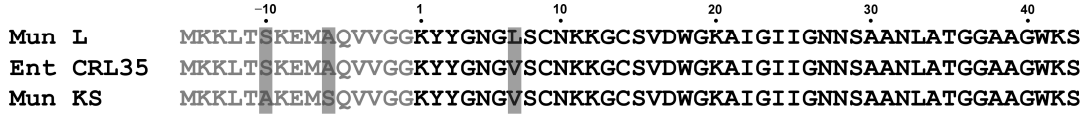

Figure 3.2. Amino acid alignment of mundticin L, enterocin CRL35 (27), and mundticin KS (21). The leader peptides are in grey. The residues that are different are highlighted.

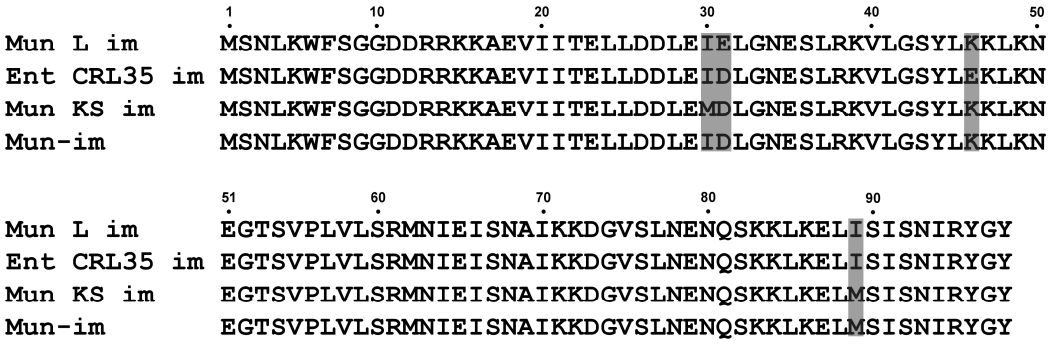

Figure 3.3. Amino acid alignment of the immunity proteins for mundticin L, enterocin CRL 35 (27), mundticin KS (21), and mundticin (18). Different amino acid residues are highlighted.

The ABC transporter for mundticin L has 674 amino acids with 98% identity to those for enterocin CRL35 and mundticin KS while the length of all three sequences are the same. It is homologous to ABC transporters for other class IIa bacteriocins, but the levels of identity are less than 50% in addition to the differences in length.

The deduced immunity protein of mundticin L was determined to be comprised of 98 amino acids (Figure 3.3). Compared to the immunity proteins of mundticin KS and enterocin CRL35, the mundticin L immunity protein is the same length but it contains different amino acids at positions 30 (Ile), 31 (Glu), 46 (Lys) and 89 (Ile); the enterocin CRL35 immunity protein contains Ile, Asp, Glu and Ile, the mundticin KS immunity protein contains Met, Asp, Lys and Met, and Mun-im (18) contains Ile, Asp, Lys and Met at the same positions.

Genetic locus of mundticin L. The structural gene of mundticin L was amplified by PCR and used to hybridize the chromosomal and plasmid DNA. The 16S rRNA gene of *E. mundtii* CUGF08 was used as the control. It was found that the mundticin L structural gene hybridized only with the plasmid DNA. Therefore, the genetic determinants of mundticin L are plasmid-associated.

N-terminal sequencing of mundticin L. The N-terminus of mundticin L was determined to be Lys-Tyr-Tyr-Gly-Asn-Gly-Leu-Ser-Xaa-Asn, which corroborates the amino acid sequence deduced from the structural gene.

DISCUSSION

Mundticin L is a new bacteriocin that is homologous to mundticin ATO6 (KS) and enterocin CRL35. A unique feature of mundticin L is the single Val-to-Leu amino acid mutation in the YGNGV motif compared to previously identified molecules mundticin ATO6, mundticin KS, and enterocin CRL35. Currently, mundticin L is the first class IIa bacteriocin that has a single amino acid difference and the difference is located in the YGNGX motif at the N-terminus which is believed to be an important region for

activity (27). The single point mutation in mundticin L may be used to examine the role of the amino acid mutation in the conserved motif.

Bacteriocin 31 (34), plantaricin C19 (2) and sakacin 5x (38) belonging to class IIa have been reported to have the YGNGL motif but have additional mutations outside of the YGNGX region. The single conservative mutation in the YGNGX motif may provide insight into the role, if any, that residue 5 of the YGNGX motif plays in the activity or spectrum of activity compared to activities mundticin ATO6 (KS) and enterocin CRL35. Although leucine and valine have similarly hydrophobicities, leucine does not have the β-methyl side chain of valine, which provides a force restraining the freedom of the ϕ and ψ torsion angles and may have an effect on the stereostructure of the bacteriocin. Whether this type of conformational change phenotypically affects the thermodynamic properties of the bacteriocin (in other words, whether the residue mutation is redundant in structure and function) is not clear. The positive electrostatic potential is polarized toward the N-terminus due to the clustered basic amino acids in the N-terminal half of the bacteriocin, which is considered essential for electrostatic tethering to the cell membrane of the target cells (4), while the function of the YGNGX consensus is not clear.

In this study, comparisons of the activity and the antimicrobial spectrum were performed for mundticin L and ATO6, but no significant differences were observed for either the level of activity or the spectrum of indicator bacteria tested (data not shown). This suggests that the fifth residue in the YGNGX complex is not essential for the activity of mundticin bacteriocins or that the difference in activity is not sufficient to be detected using traditional activity assays. Among class I bacteriocins, nisin Z differs from nisin A by one amino acid (Asn27His). The antimicrobial activities of these molecules were determined to be identical, while the diffusion rate of nisin Z is higher than that of nisin A (8), which should not be the case for mundticin L compared

to other mundticins due to the similarity of the hydrophobicities of Leu and Val. The three dimensional structures determined so far for leucocin A (13), carnobacteriocin B2 (39), Sakacin P (36) and curvacin A (17) indicates that Val is located in a β-strand-like region of the N terminus. The positive electrostatic potential is polarized toward the N-terminal region, but the role of Val as a hydrophobic residue may be different. Although studies (19, 28) indicate that the C-terminal α-helix region may be responsible for the inhibitory specificity of various bacteriocins and the N terminus may be responsible for activity, the function of the YGNGX motif has yet to be clarified.

Although mundticin L only has one residue different from other reported mundticins, the putative immunity protein and ABC transporter are also different in amino acid sequence as described above. The amino acid substitutions did not appear to affect the immunity of the mundticin L producer strain since the producer strain exhibited similar immunity levels as compared to that of the mundticin ATO6 when tested against itself. This suggests that the amino acid substitutions were not critical for the proper functioning of the immunity protein. Cross immunity tests between mundticin L and ATO6 did not reveal significant differences in the level of immunity conferred by each of the producer strains (data not shown). The difference in the sequences of immunity proteins is unusual compared to other identical or similar bacteriocins that have been identified to date. For example, pediocin PA-1 (24) and coagulin (23) differ by a single amino acid in the C-terminus, but their corresponding immunity proteins are identical. The crystal structure of the immunity protein (Mun-im) for a recently discovered mundticin produced by *E. mundtii* 15-1A was resolved (18). Mun-im has only two different residues, at positions 31 (Asp) and 89 (Met), compared to the immunity protein (Figure 3.3) for mundticin L (Glu and Ile). The Mun-im protein is a bundle of four antiparallel α helices forming a hydrophobic core,

while the cationic and anionic patches are on the surface. Compared to the three-dimensional structure of the immunity protein for enterocin A, Mun-im was found to have a similar tertiary structure despite their primary sequences are so different that the classification of these two proteins are in separate subgroups. This suggests that the difference in primary structure of the class IIa immunity proteins are highly redundant in terms of stereostructure. However, the functionality of some special motifs is worth noting, as the Mun-im protein indicated that the C-terminal IRYGY motif (Figure 3.3), which is unique for immunity proteins for the mundticin group and some other bacteriocins, is important for the immunity activity.

The organization of the genetic determinants required for mundticin L production is similar to that of mundticin KS and enterocin CRL35. DNA sequence analysis suggests that the three genes are transcribed in two operons. The gene for mundticin L is expressed in one separate operon and the genes for immunity and ABC transporter are regulated in the second operon. The promoters for mundticin L precursor and for the second operon that contains the immunity and ABC transporter were predicted to be similar to the ones confirmed by Kawamoto et al (21). The genetic organization of the mundticin group is different from the gene clusters identified for other class IIa bacteriocins. For pediocin PA-1, coagulin and plantaricin 423, the genetic determinants required for production and secretion are clustered in a single operon while the genes for other bacteriocins are contained in several operons, one for bacteriocin precursors and their immunity, one for secretion, and/or the third one for regulatory elements (10).

The genetic locus of mundticin L is plasmid-associated, which is similar to mundticin KS (21) and enterocin CRL35 (27). However, the non-coding DNA regions upstream the operons showed no homology to those of *E. mundtii* ATO6, CRL35 and NFRI 7393 (KS producer) strains. This suggests that the bacteriocin gene may have

different origins and may not come from a similar source with point mutations occurring over time.

An initial study (13) comparing the activity of pediocin PA-1, enterocin A, sakacin P, and curvacin A, found that enterocin A showed the highest antimicrobial activity against *Listeria* spp. However, all four bacteriocins were found to inhibit *Listeria* spp. at a level of parts per billion (wt/vol). More extensive studies are expected to elucidate the difference in antimicrobial activity of various class II bacteriocins including the mundticin group.

Although no listeriosis outbreaks associated with consumption of fresh alfalfa sprouts have been reported, food recalls have been made due to contamination of alfalfa sprouts with *Listeria monocytogenes* (16, 35). The potential risk of listeriosis from contaminated alfalfa sprouts cannot be underestimated. *E. mundtii* may have potential as a probiotic in select food systems and as a food-grade protective culture to improve safety and retard spoilage bacteria. *E. mundtii* CUGF08 was isolated from alfalfa sprouts, and its ability to grow during sprouting combined with its production of acid and mundticin L may prove to be useful for protecting seed sprouts by inhibition of the growth of foodborne pathogens that are associated with sprouts. Use of indigenous protective cultures isolated from sprouts to control foodborne pathogens may be more acceptable for sprout producers and their customers than preservatives or physical treatment of the finished sprouts.

Nucleotide sequence accession number. The cloned nucleotide sequence that contains the genetic determinants for mundticin L has been deposited in the GenBank database under accession number FJ899708.

REFERENCES

1. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic *streptococci*. Appl. Environ. Microbiol. **46:** 549-552.
2. **Atrih, A., N. Rekhif, A. J. G. Moir, A. Lebrihi, and G. Lefebvre.** 2001. Mode of action, purification and amino acid sequence of plantaricin C19, an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* C19. Int. J. Food. Microbiol. **68:** 93-104.
3. **Bennik, M. H. J., B. Vanloo, R. Brasseur, L. G. M. Gorris, and E. J. Smid.** 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: full characterization and interaction with target organisms. Biochim. Biophys. Acta. **1373:** 47-58.
4. **Chen, Y., R. D. Ludescher, and T. J. Montville.** 1997. Electrostatic interactions, but not the YGNGV consensus motif, govern the binding of pediocin PA-1 and its fragments to phospholipids vesicles. Appl. Environ. Microbiol. **63:** 4770-4777.
5. **Cole, C., J. D. Barber, and G. J. Barton.** 2008. The Jpred 3 secondary structure prediction server. Nucleic Acids Res. **36:** W197-W201.
6. **Collins, M. D., J. A. E. Farrow, and D. Jones.** 1986. *Enterococcus mundtii* sp. nov. Int. J. Syst. Bacteriol. **36:** 8-12.
7. **Cotter, P. D., C. Hill, and R. P. Ross.** 2005. Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. **3:** 777-788.
8. **De Vos, W. M., J. W. Mulders, R. J. Siezen, J. Hugenholtz, and O. P. Kuipers.** 1993. Properties of nisin Z and distribution of its gene, *nisZ*, in *Lactococcus lactis*. Appl. Environ. Microbiol. **59:** 213-218.
9. **De Vuyst, L., R. Callewaert, and B. Pot.** 1996. Characterization of the antagonistic activity of *Lactobacillus amylovorus* DCE 471 and large scale

isolation of its bacteriocin amylovorin L471. System. Appl. Microbiol. **19:** 9-20.

10. **Drider, D., G. Fimland, Y. Héchard, L. M. McMullen, and H. Prévost.** 2006. The continuing story of class IIa bacteriocins. Microbiol. Mol. Biol. Rev. **70:** 564-582.
11. **Dunn, S. D.** 1986. Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on western blots by monoclonal antibodies. Anal. Biochem. **157:** 144-153.
12. **Edwards, U., T. Rogall, H. Blöcker, M. Emde, and E. C. Böttger.** 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. **17:** 7843-7853.
13. **Eijsink, V. G., M. Skeie, P. H. Middelhoven, M. B. Brurberg, and I. F. Nes.** 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. **64:** 3275-3281.
14. **Gallagher, N. L. F., M. Sailer, W. P. Niemczura, T. T. Nakashima, M. E. Stiles, and J. C. Vederas**. 1997. Three-dimensional structure of leucocin A in trifluoroethanol and dodecylphosphocholine micelles: spatial location of residues critical for biological activity in type IIa bacteriocins from lactic acid bacteria. Biochem. **36:** 15062-15072.
15. **Gonzalez, C. F., and B. S. Kunka.** 1987. Plasmid-associated bacteriocin production and sucrose fermentation in *Pediococcus acidilactici*. Appl. Environ. Microbiol. **53:** 2534-2538.
16. **Gorski, L., D. Flaherty, and J. M. Duhé.** 2008. Comparison of the stress response of *Listeria monocytogenes* strains with sprout colonization. J. Food Prot. **71:** 1556-1562.
17. **Haugen, H. S., G. Fimland, J. Nissen-Meyer, and P. E. Kristiansen.** 2005.

Three-dimensional structure in lipid micelles of the pediocin-like antimicrobial peptide curvacin A. Biochem. **44:** 16149-16157.

18. **Jeon, H. J., M. Noda, Y. Matoba, T. Kumagai, and M. Sugiyama.** 2009. Crystal structure and mutagenic analysis of a bacteriocin immunity protein, Mun-im. Biochem. Biophys. Res. Commun. **378:** 574-578.
19. **Johnsen, L., G. Fimland, and J. Nissen-Meyer.** 2005. The C-terminal domain of pediocin-like antimicrobial peptides (class IIa bacteriocins) is involved in specific recognition of the C-terminal part of cognate immunity proteins and in determining the antimicrobial spectrum. J. Biol. Chem. **280:** 9243-9250.
20. **Katla, T., K. Naterstad, M. Vancanneyt, J. Swings, and L. Axelsson.** 2003. Differences in susceptibility of *Listeria monocytogenes* strains to sakacin P, sakacin A, pediocin PA-1, and nisin. Appl. Environ. Microbiol. **69:** 4431−4437.
21. **Kawamoto, S., J. Shima, R. Sato, T. Eguchi, S. Ohmomo, J. Shibato, N. Horikoshi, K. Takeshita, and T. Sameshima.** 2002. Biochemical and genetic characterization of mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. Appl. Environ. Microbiol. **68:** 3830-3840.
22. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:** 39-86.
23. **Le Marrec, C., B. Hyronimus, P. Bressollier, B. Verneuil, and M. C. Urdaci.** 2000. Biochemical and genetic characterization of coagulin, a new antilisterial bacteriocin in the pediocin family of bacteriocins, produced by *Bacillus coagulans* I_4. Appl. Environ. Microbiol. **66:** 5213-5220.
24. **Marugg, J. D., C. F. Gonzalez, B. S. Kunka, A. M. Ledeboer, M. J. Pucci, M. Y. Toonen, S. A. Walker, L. C. M. Zoetmulder, and P. A. Vandenbergh.** 1992. Cloning, expression and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. Appl.

Environ. Microbiol. **58:** 2360-2367.

25. Mengaud, J., C. Geoffroy, and P. Cossart. 1991. Identification of a new operon involved in *Listeria monocytogenes* virulence: its first gene encodes a protein homologous to bacterial metalloproteases. Infect. Immun. **59:** 1043-1049.

26. National Center for Biotechnology Information. 2009. Basic local alignment search tool. http://blast.ncbi.nlm.nih.gov/blast.cgi.

27. Saavedra, L., C. Minahk, A. P. de Ruize Holgado, and F. Sesma. 2004. Enhancement of the enterocin CRL35 activity by a synthetic peptide derived from the NH_2-terminal sequence. Appl. Environ. Microbiol. **48:** 2778-2781.

28. Salvucci, E., L. Saavedra, and F. Sesma. 2007. Short peptides derived from the NH_2-terminus of subclass IIa bacteriocin enterocin CRL35 show antimicrobial activity. J. Antimicrob. Chemocher. **59:** 1102-1108.

29. Sambrook, J., and D. W. Russell. 2001. Molecular Cloning. A Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press, New York.

30. Schägger, H. 2006. Tricine-SDS-PAGE. Nat. Protoc. **1:** 16-22.

31. Schägger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Chem. **166:** 368-379.

32. Schillinger, U., and F. Lücke. 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. **55:** 1901-1906.

33. Swiss Institute of Bioinformatics. 2009. ExPASy proteomics server. http://ca.expasy.org/.

34. Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike. 1996. Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI17. J. Bacteriol. **178:** 3585-3593.

35. **U. S. Food and Drug Administration.** 2009. Sprout recall due to potential contamination with *Listeria*. http://www.fda.gov/consumer/updates/sprouts040909.html/

36. **Uteng, M., H. H. Hauge, P. R. L. Markwick, G. Fimland, D. Mantzilas, J. Nissen-Meyer, and C. Muhle-Goll.** 2003. Three-dimensional structure in lipid micelles of the pediocin-like antimicrobial peptide sakacin P and a sakacin P variant that is structurally stabilized by an inserted C-terminal disulfide bridge. Biochem. **42:** 11417-11426.

37. **Van Hijum, S. A., and A. L. Zomer.** 2009. Prokaryotic promoter prediction. http://bioinformatics.biol.rug.nl/websoftware/ppp/ppp_start.php.

38. **Vaughan, A., V. G. H. Eijsink, T. F. O'Sullivan, K. O'Hanlon, and D. van Sinderen.** 2001. An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. J. Appl. Microbiol. **91:** 131-138.

39. **Wang, Y., M. E. Henz, N. L. F. Gallagher, S. Chai, A. C. Gibbs, L. Z. Yan, M. E. Stiles, D. S. Wishart, and J. C. Vederas.** 1999. Solution structure of carnobacteriocin B2 and implications for structure-activity relationships among Type IIa bacteriocins from lactic acid bacteria. Biochem. **38:** 15438-15447.

40. **Yang, R., M. C. Johnson, and B. Ray.** 1992. Novel method to extract large amount of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. **58:** 3355-3359.

41. **Zendo, T., N. Eungruttanagorn, S. Fujioka, Y. Tashiro, K. Nomura, Y. Sera, G. Kobayashi, J. Nakayama, A. Ishizaki, and K. Sonomoto.** 2005. Identification and production of a bacteriocin from *Enterococcus mundtii* QU 2 isolated from soybean. J. Appl. Microbiol. **99:** 1181-1190.

CHAPTER 4

HETEROLOGOUS EXPRESSION OF THE IMMUNITY PROTEIN FOR MUNDTICIN L

ABSTRACT

Mundticin L, a new class IIa bacteriocin from *Enterococcus mundtii* CUGF08 was characterized. Three genes associated with mundticin L production are organized in two operons located on a large plasmid. The gene encoding the bacteriocin precursor (munL) is contained in the first operon. The genes encoding the ABC transporter (munT) and the immunity protein (munI) are clustered in the second operon. The genetic organization of mundticin L and its homologous bacteriocins is different from other class IIa bacteriocins whose structural gene and immunity gene are usually organized in a single operon. Interestingly, deferred inhibition assay showed that the mundticin L producer was deficient in immunity to its own bacteriocin, which is unique and has never been reported for any bacteriocin. The immunity gene was amplified by PCR and cloned into the expression vector pMG36e. The immunity protein was expressed in mundticin L sensitive strains *Enterococcus faecalis* FSL 23-140 and *Listeria monocytogenes* 10403S to determine whether the immunity deficiency is due to the function of the immunity protein. The expressed immunity protein rendered FSL 23-140 and 10403S less sensitive to mundticin L but full immunity was not observed, suggesting that the immunity protein does not provide full protection. Additional factors could be involved in this unusual phenotype.

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides that inhibit related bacteria (8). Bacteriocins, especially those from lactic acid bacteria (LAB), are promising for food applications due to their "natural" character and safety for human consumption. The genetic determinants for class I and class II bacteriocins contain at least two genes: a gene for the bacteriocin precursor and a gene for the corresponding immunity protein. Additional genes may be included for transport, regulation and modification. However, for most bacteriocins, the genes for the bacteriocin precursor and the immunity protein are clustered together in a single operon. Concomitant transcription of these two genes may produce equal amounts of the bacteriocin and immunity protein so that sufficient amount of the immunity protein can protect the producer organism from being killed by its own bacteriocin. However, a recently identified group of mundticins have a unique genetic organization (2, 4, 7, 11). The gene encoding for the mundticin precursor is contained in one operon while the genes for the ABC transporter and immunity are separately organized in a downstream operon. This genetic arrangement raises the question of how the transcription of these three genes is coordinated to ensure functional immunity to provide the producer organism protection against the bacteriocin it produces.

Interestingly, we found that the mundticin L producer *Enterococcus mundtii* CUGF08 was deficient in immunity to its own bacteriocin. This phenotype is atypical and has never been reported for any bacteriocin. As an initial step toward full understanding of its molecular mechanism, the gene for the immunity protein was cloned into a Gram-positive-negative shuttle vector pMG36e and expressed in sensitive strains to determine if the immunity deficiency is due to the function of the immunity protein.

MATERIALS AND METHODS

Cultures and media. The strains and plasmids used are included in Table 4.1.

Table 4.1. Strains, plasmids, and primers used in this study.

Strains, plasmids and primers	Description	References
Strains		
E. coli DH5α	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *gal*- *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1	Invitrogen
E. faecalis FSL 23-140	Food isolate, sensitive to mundticin L	Lab strain
E. faecalis FSL 23-140A	Strain containing the plasmid pGF09	This study
L. monocytogenes 10403S	Food isolate, sensitive to mundticin L	Lab strain
L. monocytogenes 10403SA	Strain containing the plasmid pGF09	This study
Listeria ivanovii	Indicator strain for bacteriocin activity assay	Lab strain
E. mundtii CUGF08	Mundticin L producer	(4)
E. mundtii ATO6	Mundticin ATO6 producer	(2)
Plasmids		
pMG36e	Expression vector, Em^r, 3.6 kb	(14)
pGF09	pMG36e containing 312-bp *Xba*I-*Hin*dIII fragment of the immunity gene amplified by primers IMP-F and IMP-R, Em^r, 3.9 kb	This study
Primers		
IMP-F	5'-GAGGATCTAGAGATGAGTAATTTAAAG-3'	This study
IMP-R	5'-GAATAAAAGCTTCACTAATATCCATATC-3'	This study
36E-F	5'-CATCCTCTTCGTCTTGGTAGC-3'	This study
36E-R	5'-GAACCTCAACTCCAAATATCGTAG-3'	This study
Col-F	5'-GTAATCAGCAAGCGATATAC-3'	This study
Col-R	5'-GCTTCACTAATATCCATATC-3'	This study

E. coli DH5α was cultured in Luria-Bertani (LB) broth or on LB agar (Difco, Becton Dickinson and Company, Sparks, MD). LB agar and broth, both containing 200 μg/mL of erythromycin (Fisher Scientific, Fair Lawn, NJ), were used as a selective medium to screen for clones and as a medium to culture transformed *E. coli* DH5α cells for plasmid preparation, respectively. MRS broth or agar (Criterion, Santa

Maria, CA) were used for *Enterococcus mundtii* CUGF08, *E. mundtii* ATO6, *E. faecalis* FSL 23-140 and *Enterococcus faecalis* FSL 23-140A. *Listeria monocytogenes* 10403S and *L. monocytogenes* 10403SA were cultured in Brain Heart Infusion (BHI) broth or on BHI agar. Erythromycin (5 μg/mL) was used for MRS agar/broth and BHI agar/broth to screen for clones and culturing transformed strains for plasmid extraction. *Enterococcus* spp. cultured in MRS broth were incubated at 30°C without shaking. *L. monocytogenes* and *E. coli* DH5α were incubated at 37°C with shaking at 225 rpm except for cultures for plasmid preparation from *E. coli* DH5α which were incubated at 37°C with 300 rpm of shaking.

Titration of the sensitivity of wild type and immunity protein-expressing strains. Mundticin L was isolated and concentrated by a previously described pH-dependent extraction method (4). Spot diffusion assay (12) was used to titer bacteriocin activity. Prepared bacteriocin solution was subjected to two-fold dilutions in sodium phosphate buffer (pH 7.0, 50 mM) and 10 μL of each dilution were spotted onto a lawn of *Listeria ivanovii*. The lawn was prepared by overlaying 8 mL of TSB soft agar (0.75% wt/vol) which was inoculated with 50 μL of 18-h cultured *L. ivanovii*. The antimicrobial activity (AU/mL) was defined as the reciprocal of the highest dilution showing definite inhibition in the indicator lawn. Spot diffusion assay was also used to titer the sensitivity of wild type and transformed *E. faecalis* FSL 23-140 and *L. monocytogenes* 10403S strains. An adapted well diffusion assay was used to compare the sensitivities of these strains. Mundticin L (320 AU) was loaded to wells (6 mm in diameter) in agar plates. After drying, the plates were overlaid by the same method described above. Proteinase K (100 μg) was added to exclude the possibility of non-bacteriocin antimicrobial effects.

Construction of the expression vector. The immunity gene was amplified from the genomic DNA of *E. mundtii* CUGF08 using primers IMP-F and IMP-R (Table

4.1), which contained flanking *Xba*I and *Hin*dIII restriction sites, respectively. The amplified fragment and the expression vector pMG36e were digested with *Xba*I and *Hin*dIII restriction enzymes and cloned into the *Xba*I and *Hin*dIII sites of pMG36e. The recombinant pMG36e was transformed into *E. coli* DH5α by electroporation at 2.0 kV, 200 Ω and 25 μF. The vector was isolated and transformed into the sensitive strains *E. faecalis* FSL 23-140 and *L. monocytogenes* 10403S by electroporation. For transformation, electrocompetent *E. faecalis* FSL 23-140 cells were prepared by growing cells in 4% (wt/vol) glycine according to the method described by Shepard et al., (13); electrocompetent *L. monocytogenes* 10403S cells were made by treatment with 10 μg/mL of penicillin G (Sigma, St. Louis, MO) (10). Electroporation (Gene Pulser II, Bio-Rad, Hercules, CA) was used to introduce the expression vector. Prior to electroporating the recipient strains, sequencing of the insert from the flanking regions of pMG36e with primers 36E-F and 36E-R (Table 4.1) was done to ensure that the sequence and reading frame of the insert was correct. The expression of the immunity gene was confirmed by plasmid profiling, sensitivity testing to mundticin L, and PCR amplification of the immunity gene using primers Col-F and Col-R.

The genomic DNA of *E. mundtii* CUGF08 was isolated by a chloroform/phenol extraction method (9). Plasmids from *E. mundtii* CUGF08 were isolated by a rapid lysis method (1). The expression vector pMG36e and the subsequent recombinant plasmid pGF09 were isolated from *E. coli* DH5α using a plasmid mini kit (Qiagen, Germantown, MD) according to the procedures described by the manufacturer. The expression vector pGF09 from *E. faecalis* FSL 23-140A and *L. monocytogenes* 10403SA was isolated by the Qiagen mini kit with adjustment: lysozyme (Sigma) and mutanolysin (Sigma) were added to resuspension buffer to concentrations of 5 mg/mL and 15 U/mL, respectively (5). PCR reactions were performed in a RoboCycler (Stratagene, La Jolla, CA) at annealing temperature of 37°C for 30 reactions.

Restriction enzymes were supplied by Fisher Scientific. All oligonucleotides were synthesized and supplied by Integrated DNA Technologies (Coralville, IA). Nucleotide sequencing was performed at Cornell University Life Sciences Core Laboratories.

RESULTS AND DISCUSSION

Heterologous expression of the immunity protein. The mundticin L producer *E. mundtii* CUGF08 displayed an immunity deficiency to its own bacteriocin. When 320 AU of mundticin L was added to a 6-mm well in MRS agar, a clear inhibition zone against *E. mundtii* CUGF08 was observed (Figure 4.1A). Proteinase K digested the bacteriocin and the producer was not inhibited (Figure 4.1B). This suggests that the producer is naturally deficient in immunity to mundticin L. This phenotype has not been reported for any bacteriocin. Agar diffusion assay of mundticin ATO6, a similar bacteriocin produced by *E. mundtii* ATO6 (2), showed that ATO6 also lacked immunity to its bacteriocin (data not shown). Therefore, the immunity deficiency appears to be unique to the mundticin producers.

Heterologous expression of the immunity protein for mundticin L was performed to determine whether the immunity deficiency was due to the function of the immunity protein or additional extraneous host factors such as endogenous proteases. The gene encoding the immunity protein was transcriptionally and translationally fused into the multiple cloning site of pMG36e. The protein was successfully expressed in sensitive strains of *L. monocytogenes* 10403S and *E. faecalis* FSL 23-140, as confirmed by plasmid profiling and phenotypic change of the sensitivity of the transformed strains. Transformed strains of *L. monocytogenes* 10403SA and *E. faecalis* FSL 12-140A that expressed the immunity protein displayed less sensitivity compared to that of the wild type strains at the same mundticin L concentrations (Figure 4.1C, D, E and F). Slightly smaller inhibition zones were observed with strains 10403SA and FSL 12-140A.

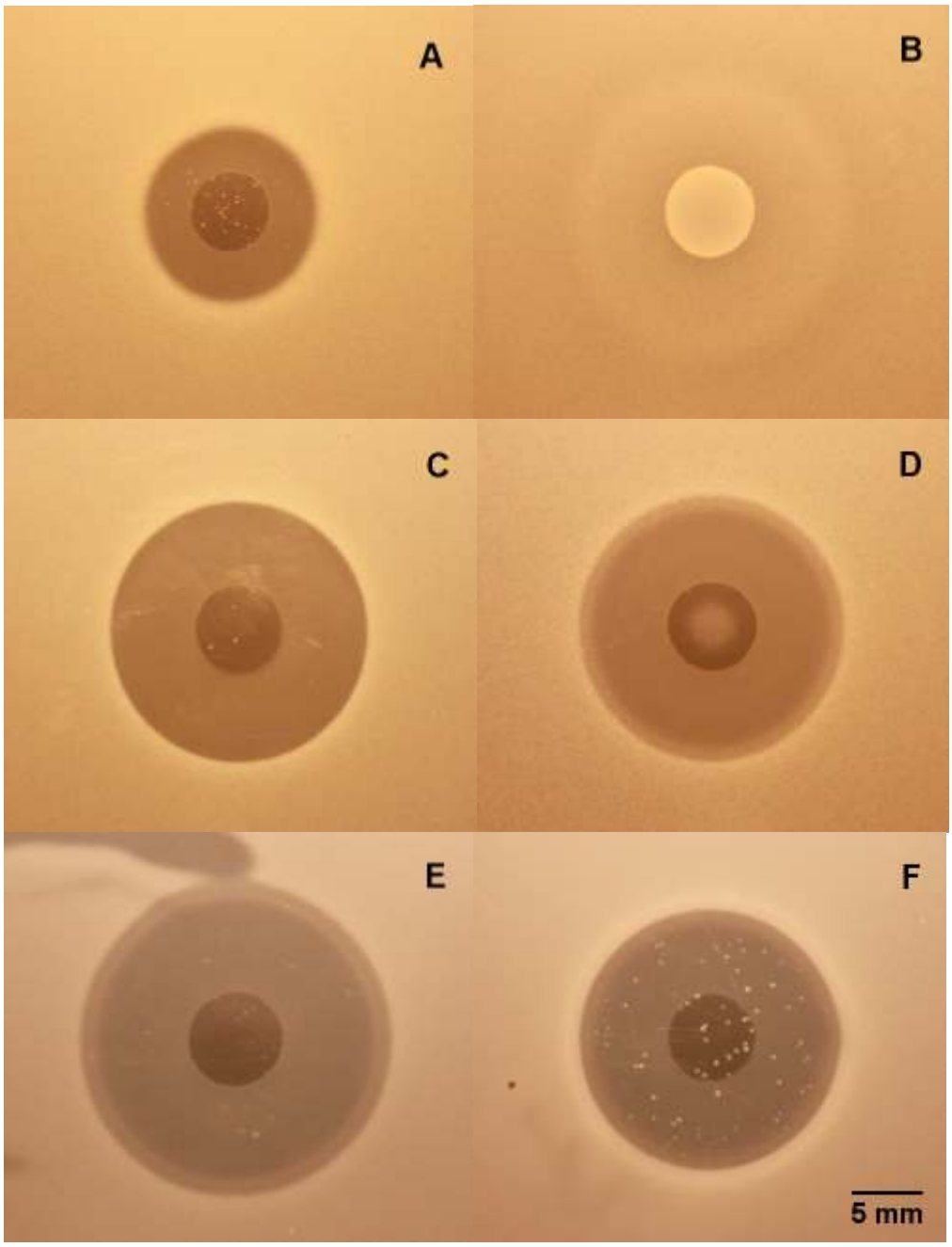

Figure 4.1. Sensitivity of different strains to mundticin L. Each well was loaded with 320 AU mundticin L. *E. mundtii* CUGF08 was deficient in immunity to its own bacteriocin (A) while self-inhibition disappeared after adding proteinase K (100 μg) to the well (B); Sensitivity of the wild type *E. faecalis* FSL 23-140 (C), *L. monocytogenes* 10403S (E) and the immunity protein-expressing strains FSL 23-140A (D) and 10403SA (F) were similar. However, titration of the sensitivity of wild type and transformed strains was different (see Table 4.2).

However, both strains still remained sensitive to mundticin L. It is clear that the difference in the size of inhibition zones displayed by the wild type and immunity-expressing strains was not significant, suggesting the immunity protein did not provide full protection for the sensitive strains.

Titration of the sensitivity of wild type and immunity protein-expressing strains. To quantitatively differentiate wide type and immunity-expressing strains, the sensitivity of these strains was titrated using the spot diffusion assay (Table 4.2). *L. monocytogenes* 10403SA was determined to be four times less sensitive to mundticin L than the wild type strain while *E. faecalis* FSL 23-140A was two times less sensitive than the wild type strain. The observed difference in sensitivity confirmed the function of the immunity protein. However, the immunity protein conferred only partial protection to the sensitive strains tested.

Table 4.2. Minimum inhibitory concentration of wild type and immunity protein-expressing strains to mundticin L.

Bacterial strains	Sensitivity (AU/mL)
E. faecalis FSL 23-140	1600
E. faecalis FSL 23-140A	3200
L. monocytogenes 10403S	400
L. monocytogenes 10403SA	1600

Heterologous expression of the immunity protein for mundticin L showed that the immunity protein did not confer full protection to sensitive strains, suggesting that the protein may function in a similar manner as that in the producer organism *E. mundtii* CUGF08. However, *E. mundtii* CUGF08 is capable of growing to typical high cell densities in liquid culture such us MRS broth (data not shown), suggesting that the growth of the producer organism was not inhibited by the production of mundticin L

in liquid culture. Certain unknown molecular factors may diffuse more readily to be accessible to planktonic cells in liquid culture while their diffusion could be limited due to the character of the agar medium. Additional uncharacterized factors may be involved in immunity to mundticin L. Therefore, *E. mundtii* CUGF08 must have a mechanism to protect itself from being killed by its bacteriocin. This mechanism may involve multiple factors besides the immunity protein. Random mutagenesis of the wild type strain may elucidate additional genes involved in mundticin L immunity responsible for this unique phenotype.

It should be noted that the immunity gene in the pMG36e was a translational fusion; i.e. the product of translation may contain additional N-terminal residues that are encoded by the upstream fragment of the expression vector. Therefore, the N-terminal residues in addition to the 98 amino acids in the original immunity protein might have an effect on the phenotypes of the strains 10403S and FSL 23-140A. However, it should not be a determining factor in immunity because the expression of bacteriocin immunity proteins by translationally fusing into the promoter region and translational start codon of pMG36s has been studied before (5, 6). These studies similarly fused the immunity gene into the same reading frame of pMG36e. The immunity proteins were successfully expressed and conferred full immunity to sensitive strains. Therefore, the N-terminal fusion should not be a determining factor for the immunity phenotype in the transformed strains from this study.

The genes for mundticin L immunity protein and mundticin L structural gene were contained in separate operons, which is unique for mundticin L, KS, ATO6 and Enterocin CRL35. This unique genetic organization was confirmed by a transcriptional study of mundticin KS (7). This lack of translational coupling raises the question as to how expression of the bacteriocin and the immunity protein are coordinated at the transcription level to ensure that sufficient levels of the immunity

protein are produced for full immunity. It may be possible that the immunity deficiency was due to the unbalanced expression of these two genes. As a possible means to determine this, engineered expression vectors may be constructed with both genes in a single operon, in order to determine whether the immunity deficiency was due to differing levels of mundticin L and its immunity protein. Initial attempts for the proposed construct using pGKV259 (15) were unsuccessful. Unfortunately, the cloning host *E. coli* DH5α containing the constructed vector could not grow well (data not shown), possibly due to the intracellular toxicity generated by the expressed bacteriocin. Evaluation of the mundticin L and immunity gene transcripts will confirm whether the expected transcript and expression levels are being synthesized.

However, additional factors may be involved in the immunity deficiency. An investigation of the man-PST operon in *E. mundtii* CUGF08 and its expression level may be worthwhile since high level of expression may result in producer sensitivity to mundticin L (3). A genome-wide mutagenesis may be the best strategy to identify potential additional genetic factors for this interesting phenotype.

REFERENCES

1. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic *Streptococci*. Appl. Environ. Microbiol. **46:**549-552.
2. **Bennik, M. H., B. Vanloo, R. Brasseur, L. G. Gorris, and E. J. Smid.** 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: full characterization and interaction with target organisms. Biochim. Biophys. Acta. **1373:**47-58.
3. **Diep, D. B., M. Skaugen, Z. Salehian, H. Holo, and I. F. Nes.** 2007. Common mechanisms of target cell recognition and immunity for class II bacteriocins. Proc. Natl. Acad. Sci. U. S. A. **104:**2384-2389.
4. **Feng, G., G. K. Guron, J. J. Churey, and R. W. Worobo.** 2009. Characterization of mundticin L, a class IIa anti-Listeria bacteriocin from *Enterococcus mundtii* CUGF08. Appl. Environ. Microbiol. **75:**5708-5713.
5. **Fimland, G., V. G. Eijsink, and J. Nissen-Meyer.** 2002. Comparative studies of immunity proteins of pediocin-like bacteriocins. Microbiology. **148:**3661-3670.
6. **Franz, C. M., M. J. van Belkum, R. W. Worobo, J. C. Vederas, and M. E. Stiles.** 2000. Characterization of the genetic locus responsible for production and immunity of carnobacteriocin A: the immunity gene confers cross-protection to enterocin B. Microbiology. **146:**621-631.
7. **Kawamoto, S., J. Shima, R. Sato, T. Eguchi, S. Ohmomo, J. Shibato, N. Horikoshi, K. Takeshita, and T. Sameshima.** 2002. Biochemical and genetic characterization of mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. Appl. Environ. Microbiol. **68:**3830-3840.

8. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39-85.
9. **Mengaud, J., C. Geoffroy, and P. Cossart.** 1991. Identification of a new operon involved in *Listeria monocytogenes* virulence: its first gene encodes a protein homologous to bacterial metalloproteases. Infect. Immun. **59:**1043-1049.
10. **Park, S. F., and G. S. Stewart.** 1990. High-efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. Gene. **94:**129-132.
11. **Saavedra, L., C. Minahk, A. P. de Ruiz Holgado, and F. Sesma.** 2004. Enhancement of the enterocin CRL35 activity by a synthetic peptide derived from the NH2-terminal sequence. Antimicrob. Agents Chemother. **48:**2778-2781.
12. **Schillinger, U., and F. K. Lucke.** 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. **55:**1901-1906.
13. **Shepard, B. D., and M. S. Gilmore.** 1995. Electroporation and efficient transformation of *Enterococcus faecalis* grown in high concentrations of glycine. Methods Mol. Biol. **47:**217-226.
14. **van de Guchte, M., J. M. van der Vossen, J. Kok, and G. Venema.** 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55:**224-228.
15. **van der Vossen, J. M., D. van der Lelie, and G. Venema.** 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. **53:**2452-2457.

CHAPTER 5

BACTERIOCINOGENIC LACTIC ACID BACTERIA AS BIOLOGICAL CONTROL AGENTS TO ENHANCE THE SAFETY OF SPROUTS

ABSTRACT

Alfalfa sprouts are a well known food vehicle for human pathogens and have been responsible for numerous foodborne illness outbreaks. A biological control strategy using food-grade protective cultures may be promising in preventing pathogens from rapidly growing to high levels during sprouting. Unlike single-step treatment with surface sanitizers such as calcium hypochlorite which may allow survival of pathogens trapped inside of the seeds and thus rapid growth during subsequent sprouting process, protective cultures provide sustainable antimicrobial effect during the whole process of sprouting by acid production, bacteriocin and exclusive competition for growth. *Enterococcus mundtii* CUGF08 and *Lactococcus lactis* AA4 are two bacteriocinogenic lactic acid bacteria (LAB) isolated from alfalfa sprouts. *E. mundtii* CUGF08 produces mundticin L, a class IIa bacteriocin which has especially high antimicrobial activity against *Listeria* spp. *L. lactis* AA4 produces nisin Z. These two lactic acid bacteria were inoculated to alfalfa seeds which were pre-inoculated with different levels of *Salmonella enterica*, *L. monocytogenes* and *Escherichia coli* O157:H7. The growth of *E. mundtii* CUGF08, *L. lactis* AA4, *S. enterica*, *L. monocytogenes* and *E. coli* O157:H7 on alfalfa sprouts were monitored over the course of sprouting. Both isolates controlled the growth of pathogens to a significant extent. In general, *L. lactis* AA4 showed a higher efficacy than *E. mundtii* CUGF08 for controlling the growth of *L. monocytogenes* and *S. enterica*, and lower inhibition against *E. coli* O157:H7.

INTRODUCTION

Alfalfa sprouts have been associated with many foodborne illness outbreaks around the world. Contaminated seeds have been identified as the source for most of the reported outbreaks (10). A systematic approach with multiple hurdles for the entire sprout production process could be an effective strategy for reducing the risks of sprout-associated outbreaks. This approach would integrate good agricultural practices (GAP) to reduce contamination of seeds pre-harvest, seed decontamination methods to eliminate pathogens from contaminated seeds and/or sprout treatment to decontaminate sprouts during the sprouting process. Seed treatment is the preferred strategy compared to sprout treatment because the contamination levels are lower in the seeds and an organoleptic change in the finished sprouts is less likely. Many studies have investigated physical, chemical, and biological intervention methods to decontaminate or eliminate pathogens from alfalfa seeds. None of the seed treatment methods have been shown to be effective in eliminating pathogens from seeds (> 5-log) without compromising the seed viability. Although FDA currently recommends seeds be treated in 20,000 ppm calcium hypochlorite to mitigate the situation, evidence has shown that outbreaks have been linked to seeds that were treated by this method (14). Alfalfa seeds are relatively small and thus higher in surface area than other types of seeds, such as mung bean. The surface of seeds has crevices and wrinkles where pathogens can be trapped inside and become inaccessible to surface sanitizers (5). These special structures may explain why alfalfa seeds are the main type of seeds that have been associated with foodborne illness outbreaks. Therefore, development of decontamination methods whose efficacy is not limited by the surface structure of seeds is needed. Among them, physical methods such as thermal treatment and high pressure that can inactivate pathogens regardless of seed structure have shown potential (2, 9, 11, 12).

Biological control of pathogen growth during sprouting may have potential since the antimicrobial effect due to acid and bacteriocin production, and competitive exclusion would be sustainable during the whole process of sprouting. Since protective cultures exert antimicrobial effects during seed sprouting, they may inhibit pathogens regardless of seed structures. Although we have shown that thermal treatment of alfalfa seeds may achieve 5-log reduction without significantly reducing germination rates of alfalfa seeds (6), post-processing contamination during sprouting may pose additional risks. A few studies have investigated the effectiveness of potential protective cultures in inhibiting pathogens on alfalfa sprouts. Plant-associated *Pseudomonas fluorescens* 2-79 was shown to successfully control the growth of *S. enterica* strains on alfalfa sprouts for the first 6 days of sprouting (8). However, the effect of this organism on *E. coli* O157:H7 was not investigated despite it has been responsible for numerous sprout associated foodborne illness outbreaks. Although pseudomonads are part of the natural microbiota in sprouted seeds, the use of pseudomonads as protective cultures may be controversial due to their non-food-grade status. Lactic acid bacteria would be the best applicable protective cultures due to their generally recognized as safe (GRAS) status and their potential in antibiosis. *E. mundtii* ATO6 produces mundticin ATO6 which is a class IIa bacteriocin that exhibits high inhibitory activity against *L. monocytogenes* (4). The potential of *E. mundtii* ATO6 and two additional bacteriocinogenic *Pediococcus parvulus* strains as protective cultures to control the growth of *L. monocytogenes* in mung bean sprouts has been investigated (3). The specific growth rate of *E. mundtii* ATO6 was higher than that of *Pediococcus parvulus* under modified atmosphere storage with elevated CO_2. However, at refrigeration temperatures the growth of *L. monocytogenes* on mung bean sprouts was not inhibited by *E. mundtii* ATO6. The growth control of *E. mundtii* ATO6 on *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157:H7 during sprouting

was not studied. The inhibitory effect of *Lactococcus lactis* on the growth of *L. monocytogenes* during sprouting of alfalfa seeds was studied (13). Although *L. lactis* showed significant inhibition on *L. monocytogenes* compared to the control, the pathogen levels on growing sprouts was still higher than the initial inoculation level.

Lactic acid bacteria have also been isolated from alfalfa sprouts and screened for antagonism against relevant pathogens associated with sprouts (17). *Lactococcus lactis* subsp. *lactis* was shown to be the most effective isolate for inhibiting *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157:H7. Although *L. lactis* showed significant inhibition on all three pathogens, the study was performed in model systems rather than on growing sprouts.

Although the aforementioned studies investigated the potential of LAB such as *L. lactis*, *E. mundtii* and *Pediococcus parvulus* as biocontrol agents to enhance sprout safety, none of them studied their efficacy on all three relevant pathogens with growing alfalfa sprouts. Research endeavors have been focused on the biocontrol of *L. monocytogenes* which is less of a concern for sprout safety. Moreover, most studies used LAB that were isolated from other sources rather than LAB species that are part of the natural microbiota of seed sprouts. Application of sprout-associated LAB isolates for sprouts as protective cultures may be more acceptable by consumers and also effective in inhibiting relevant pathogens due to their possible physiological and genetic adaptation to sprouts. We isolated two bacteriocinogenic lactic acid bacteria strains from fermented alfalfa sprouts: *E. mundtii* CUGF08 and *L. lactis* AA4. *E. mundtii* CUGF08 produces mundticin L, an anti-*Listeria* bacteriocin which has been characterized in a previous study (7); *L. lactis* AA4 produces nisin Z. Nisin Z and mundticin L are typical bacteriocins representing class I and class IIa bacteriocins, respectively. This study investigated the potential of these two bacteriocinogenic LAB as biological control agents to inhibit the growth of *L. monocytogenes*, *S. enterica* and

E. coli O157:H7 on growing alfalfa sprouts.

MATERIALS AND METHODS

Cultures and media. Two LAB strains isolated from fermented alfalfa sprouts (2% NaCl and reduced oxygen environment) were used as protective cultures: *E. mundtii* CUGF08 and *L. lactis* AA4. *E. mundtii* CUGF08 is a mundticin L-producer which was described in a previous study (7); *L. lactis* was found to produce nisin Z which was identified by PCR and sequencing of the nisin gene using primers and reaction conditions described elsewhere (16). The isolated strains were identified by sequencing amplified 16S rRNA genes and homology analysis using the BLAST program described previously (7). A cocktail of five strains of *L. monocytogenes*, *E. coli* O157:H7, and five serovars of *S. enterica* were used for inoculation of alfalfa seeds. The detailed information about the bacterial strains used in this study was included in Table 5.1. To facilitate enumeration of *S. enterica* and *E. coli* O157:H7, all ten strains were transformed with pGFP (Clontech Laboratories, Palo Alto, CA), a plasmid encoding a green fluorescent protein and ampicillin resistance. Cultures were stored at -80°C in respective media containing 15% glycerol.

To prepare the inocula, *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth (Difco, Becton Dickinson and Company, Sparks, MD). Tryptic soy broth (TSB) (Criterion, Santa Maria, CA) supplemented with 100 μg/ml of ampicillin (Fisher Scientific, Fair Lawn, NJ) was used to culture *S. enterica* and *E. coli* O157:H7 for the inoculated studies. All pathogens were grown at 37°C with shaking at 225 rpm (Innova 4230, New Brunswick Scientific, Edison, NJ). MRS broth (Criterion) was used to culture *E. mundtii* CUGF08 and *L. lactis* AA4 at 30°C without aeration.

For microbiological enumeration of the inoculated alfalfa seeds and sprouts, Oxford medium base with Oxford antimicrobial supplement (Difco) was used to culture

Table 5.1. Bacterial strains used in this study.

Bacterial strains	Description	Sources
L. lactis AA4	Protective culture, nisin z producer	Isolate from alfalfa sprouts
E. mundtii CUGF08	Protective culture, mundticin L producer	Isolate from alfalfa sprouts
L. ivanovii	Indicator strain for bacteriocin activity assay	Lab strain
L. monocytogenes 19112	Pathogen for inoculation	M. Wiedmann, Cornell University
L. monocytogenes 1043S	Pathogen for inoculation	M. Wiedmann, Cornell University
L. monocytogenes F8027	Pathogen for inoculation	M. Wiedmann, Cornell University
L. monocytogenes F8309	Pathogen for inoculation	M. Wiedmann, Cornell University
L. monocytogenes J1-119	Pathogen for inoculation	M. Wiedmann, Cornell University
S. enterica Montevideo	Pathogen for inoculation, harboring pGFP with Amp^r	Lab strain
S. enterica Gaminara	Pathogen for inoculation, harboring pGFP with Amp^r	Lab strain
S. enterica Rubislaw	Pathogen for inoculation, harboring pGFP with Amp^r	Lab strain
S. enterica Hartford	Pathogen for inoculation, harboring pGFP with Amp^r	Lab strain
S. enterica Typhimurium	Pathogen for inoculation, harboring pGFP with Amp^r	Lab strain
E. coli O157:H7 ATCC 35150	Pathogen for inoculation, harboring pGFP with Amp^r	Clinical isolate (J. Russell, Cornell University)
E. coli O157:H7 ATCC 43895	Pathogen for inoculation, harboring pGFP with Amp^r	Raw Hamburger meat isolate (M. P. Doyle, University of Georgia)
E. coli O157:H7 933	Pathogen for inoculation, harboring pGFP with Amp^r	Raw Hamburger meat isolate (M. P. Doyle, University of Georgia)
E. coli O157:H7 ATCC 43894	Pathogen for inoculation, harboring pGFP with Amp^r	Clinical isolate (M. P. Doyle, University of Georgia)
E. coli O157:H7 ATCC 43889	Pathogen for inoculation, harboring pGFP with Amp^r	Clinical isolate (M. P. Doyle, University of Georgia)

L. monocytogenes. Tryptic soy agar (TSB containing 1.5% agar) with 100 μg/ml of ampicillin was used for culturing *S. enterica* and *E. coli* O157:H7. *E. mundtii* CUGF08 was grown on MRS agar containing 0.02% (wt/vol) sodium azide (Sigma, St. Louis, MO) for samples inoculated with *L. monocytogenes* or on enterococcosel agar (EA) for samples inoculated with *S. enterica* or *E. coli* O157:H7. *L. lactis* AA4 was grown on Alsan agar which is a highly selective medium for *L. lactis* subsp. *lactis* and was prepared according to the procedures described by Alzoreky et al (1).

Inoculation of alfalfa seeds with pathogens and protective cultures. Alfalfa seeds (Lucerne) were supplied by Sringwater Sprouts, Honeoye Falls, NY. Protective cultures were inoculated to alfalfa seeds which were pre-inoculated with *L. monocytogenes*, *S. enterica*, or *E. coli* O157:H7. The artificially contaminated seeds were subjected to four treatments with protective cultures: single culture of *E. mundtii* CUGF08, single culture of *L. lactis* AA4, co-culture of *E. mundtii* CUGF08 and *L. lactis* AA4, and the control (no inoculation of either culture).

For inoculation of alfalfa seeds with pathogens at high levels, *L. monocytogenes* strains were grown in 5 ml of BHI broth to log phase (OD_{600nm}=0.9) for 18 h. One milliliter of each strain was centrifuged at 6000 ×*g* at 4°C for 10 min. The cells were washed once with 1 ml of sterile Milli-Q water and resuspended in sterile Milli-Q water to remove any growth media and by-products. The resuspended cells were combined to make a 5 ml of cocktail *L. monocytogenes* and mixed by low-speed vortexing. One milliliter of the mixed cocktail was diluted 1,000 fold and five milliliters of the highest dilution was added to 1 kg of alfalfa seeds combined with 1.5 L sterile Milli-Q water. The seeds were soaked for 20 min with agitation to ensure even inoculation. The seeds were transfer to multiple layers of sterile cheese cloth on top of two sterile wire racks (0.2 m^2 total in area) and dried for 24 h in a biological safety cabinet. To prepare inocula of *S. enterica* and *E. coli* O157:H7, the same

procedure was used except the growth medium was TSB supplemented with ampicillin.

To prepare inocula of protective cultures, *E. mundtii* CUGF08 and *L. lactis* AA4 were grown in 5 ml of MRS broth for 8 h. One milliliter of each culture was separately inoculated into duplicate 250 ml of MRS broth and incubated for 16 h to log phase (OD_{600nm}=0.6). The cells were centrifuged at 6000 ×*g* at 4°C for 15 min and washed once in an equal volume of Milli-Q water. The washed cells were resuspended in 200 mL of water and combined to a final volume of 400 ml for each culture. Alfalfa seeds that were pre-inoculated with *L. monocytogenes*, *S. enterica*, or *E. coli* O157:H7 were inoculated with single bioprotective culture of *E. mundtii* CUGF08, *L. lactis* AA4, or mixed cultures of both. To inoculate seeds with a single culture, 200 ml of *E. mundtii* CUGF08 or *L. lactis* AA4 resuspended cells were mixed with 250 ml Milli-Q water to make 450 ml total and approximately 200 g of artificially contaminated seeds. For co-culture inoculation, 200 ml of *E. mundtii* CUGF08 and *L. lactis* AA4 were combined and mixed with 50 ml of sterile Milli-Q water to make a 450 ml mixture that was mixed with approximately 200 g of contaminated seeds. For the control, 450 ml of sterile Milli-Q water was mixed with 200 g of contaminated seeds. All mixtures were agitated occasionally for 20 min at room temperature (22 ± 2°C).

Germination of alfalfa seeds. Approximately 50 g of freshly inoculated alfalfa seeds from each of the four treatments was transferred to a sterile aluminum tray [0.527 m (L) × 0.325 m (W) × 0.086 m (H)] (BJWC, Natick, MA). The trays were lined with a layer of urethane foam (Carpenter Co., Russellville, KY) and a sheet of filter paper (3M filter paper; Fisher Scientific) on top. The seeds were spread evenly to a single layer over the filter paper and covered with aluminum foil. The trays were incubated at 30°C for 4 days. Except for day 0, sprouting seeds in each tray were sprayed with 20 ml of sterile Milli-Q water once a day using a plant sprayer (Misco

Enterprises, Dunellen, NJ) right after sampling for bacterial analysis.

Bacterial analysis. Growth of pathogens and bioprotective cultures were monitored on alfalfa seeds and sprouts during the sprouting process from day 0 to day 4. 25 g of seeds or sprouts were sampled for bacterial analysis. Seeds or sprouts were diluted 10 fold with 0.1% (wt/vol) sterile peptone (Difco) water and stomached at 260 rpm by a stomacher (Stomacher 400, Seward Ltd., Basingstoke, UK) for 1 min. Stomached samples were subjected to a series of 10-fold dilution in sterile peptone water. Selected dilutions were surface-plated onto selective media. Oxford medium base plus antimicrobial supplement was used to select for *L. monocytogenes*. TSA plus ampicillin was used for *S. enterica* and *E. coli* O157:H7. Alsan medium was used to select for *L. lactis* subsp. *lactis*. EA was used to select for *Enterococcus* spp. except for the seeds pre-inoculated with *L. monocytogenes* which can grow along with *Enterococcus* spp. on this medium. Instead, MRS plus 0.02% sodium azide was used to estimate *E. mundtii* CUGF08 when seeds were inoculated with *L. monocytogenes*. All media were tested for their growth selectivity of the intended microorganism. Oxford medium and MRS plus sodium azide were incubated at 30°C for 48 h prior to enumeration. EA and TSA plus ampicillin were incubated at 37°C for 24 h. Alsan medium was incubated at 30°C for 48 h in anaerobic jars (Difco). GasPak EZ Anaerobe Container System and catalyst (Becton Dickinson and Company, Sparks, MD) were placed in the jars to produce anaerobic conditions which were monitored by the inclusion of an anaerobic indicator (Oxoid, Hants, United Kingdom).

Bacteriocin activity assay and pH of finished sprouts. Bacteriocin activity of the sprouts was measured using a spot diffusion assay (15). Finished sprouts were sampled, diluted 10 fold and stomached as described above. The mixed solution was subjected to 2-fold dilutions in sodium phosphate buffer (pH 7.0, 50 mM) and 10 μL of each dilution was spotted onto a lawn of *Listeria ivanovii*, the sensitive bacteriocin

indicator strain. The lawn was prepared by overlaying 8 mL of TSB soft agar (0.75% wt/vol) which was inoculated with 50 µL of 18-h cultured *L. ivanovii*. The antimicrobial activity (AU/mL) was defined as the reciprocal of the highest dilution showing definite inhibition in the indicator lawn. The pH was measured on day 4. 25 g of sprouts were diluted 10 fold and stomached prior to pH measurement (Orion 2 Star, Thermo Scientific, Waltham, MA).

Statistics. All four treatments were performed in triplicate. Bacterial analysis was performed in duplicate plating for each trial. The data points are presented as averages of three trials. JMP 8.0 (SAS, Cary, NC) was used to perform statistical analysis. Type I error (*P*) was 0.05 by default.

RESULTS AND DISCUSSION

Inhibition of pathogens on alfalfa sprouts by protective cultures. When singly inoculated onto alfalfa seeds, both *E. mundtii* CUGF08 and *L. lactis* AA4 significantly inhibited the growth of *L. monocytogenes* compared to the control (Figure 5.1). However, *L. lactis* AA4 showed greater inhibition of *L. monocytogenes* growth compared to that observed with *E. mundtii* CUGF08. When both AA4 and CUGF08 were inoculated onto alfalfa seeds, the inhibition of *L. monocytogenes* was similar to that of single inoculation of AA4, suggesting that a synergy of AA4 and CUGF08 is not readily apparent. In the control, *L. monocytogenes* rapidly grew from 5-log CFU/g to higher than 8-log CFU/g within 24 h of sprouting without any protective cultures. Although *L. lactis* AA4 and the combination of protective cultures slowed the growth of *L. monocytogenes*, the level of the pathogen on the finished sprouts was still greater than 1-log higher than the initial inoculation level.

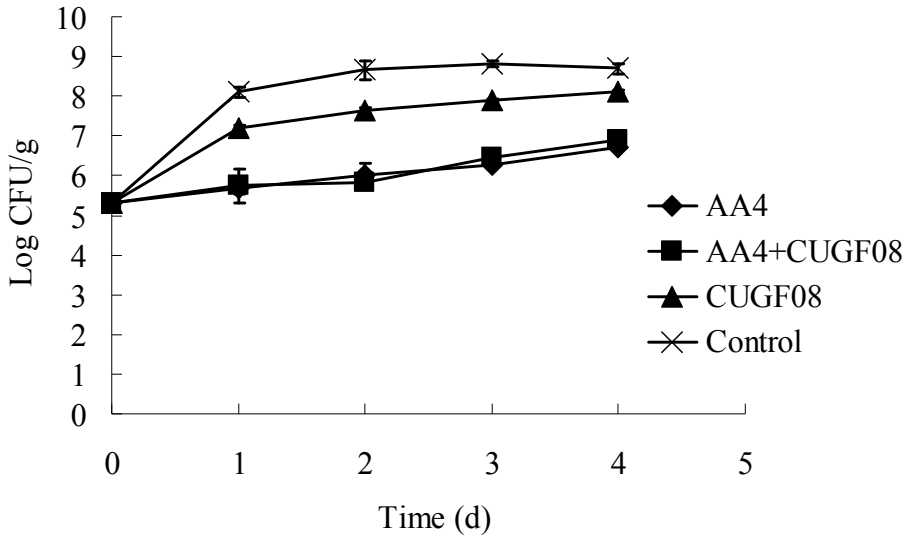

Figure 5.1. Growth of *Listeria monocytogenes* on alfalfa sprouts inoculated with *Lactococcus lactis* AA4, *Enterococcus mundtii* CUGF08, both, and none (control).

The growth of *S. enterica* was inhibited by both protective cultures (Figure 5.2). Either single inoculation of *E. mundtii* CUGF08 or *L. lactis* AA4, or both, controlled the growth of *S. enterica* to a significant extent, but less pronounced than *L. monocytogenes*. Unlike what was observed with bio-control of *L. monocytogenes*, co-inoculation of both protective cultures showed the most significant inhibition with *S. enterica* compared to the single inoculation of either culture. Single inoculation of *L. lactis* AA4 inhibited *S. enterica* more than that observed with *E. mundtii* CUGF08 inoculated seeds. When compared to the control, which maintained the highest level of *S. enterica* on sprouts, *E. mundtii* CUGF08 still showed significant growth inhibition of *S. enterica*. Single inoculation of *L. lactis* AA4 or co-inoculation with *E. mundtii* CUGF08, allowed for an increase of greater than 1-log of *S. enterica* levels, while the control with no protective cultures added, allowed for *S. enterica* to increase by more than 2-logs.

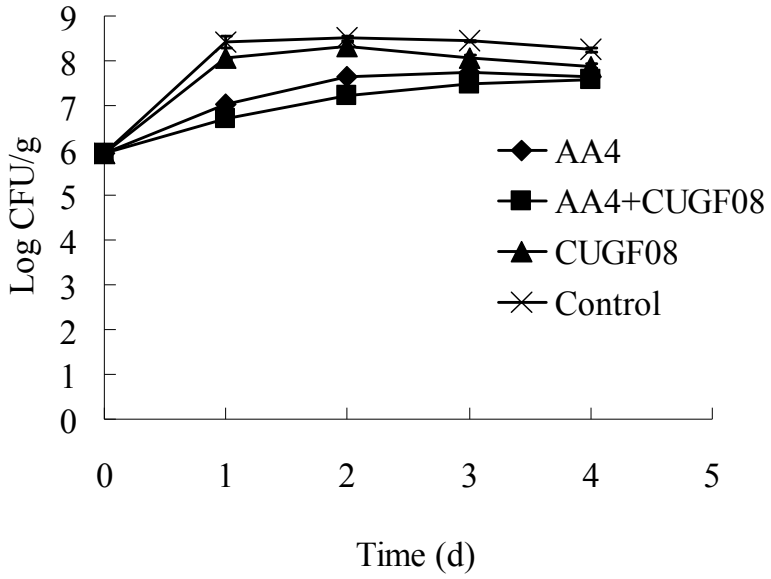

Figure 5.2. Growth of *Salmonella enterica* on alfalfa sprouts inoculated with *Lactococcus lactis* AA4, *Enterococcus mundtii* CUGF08, both, and none (control).

Although the inoculation level of *E. coli* O157:H7 on alfalfa seeds was slightly lower than the other two pathogens tested, *E. coli* O157:H7 grew to a comparable level within the first 24 h on seeds that were not inoculated with any protective cultures (Figure 5.3). Neither single culture of *L. lactis* AA4 nor co-culture controlled the growth of the pathogen whose levels were even higher than the control. However, *E. mundtii* CUGF08 showed the most significant inhibition of *E. coli* O157:H7 from day 2 to day 4. The finished sprouts inoculated with *E. mundtii* CUGF08 contained the lowest pathogen population. The results for inhibition of *E. coli* O157:H7 was different compared to *L. monocytogenes* and *S. enterica,* with regards to the protective culture showing the most significant potential. This may be due to the fact that *E. coli* O157:H7 is naturally more acid-resistant than the other two pathogens. Therefore, *L. lactis* AA4 is weaker in controlling *E. coli* O157:H7. The mechanism for the antibiosis of *E. mundtii* CUGF08 against *E. coli* O157:H7 must be different from that of *L. lactis* AA4.

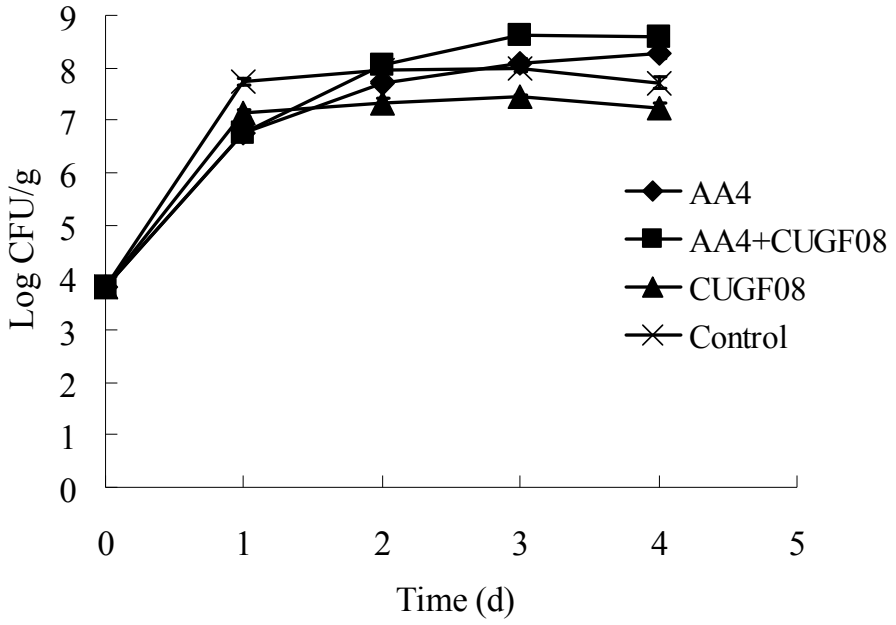

Figure 5.3. Growth of *E. coli* O157:H7 on alfalfa sprouts inoculated with *Lactococcus lactis* AA4, *Enterococcus mundtii* CUGF08, both, and none (control).

Growth of protective cultures on alfalfa sprouts. The growth curves of protective cultures on sprouts that were inoculated with *L. monocytogenes*, *S. enterica* or *E. coli* O157:H7 are similar and reported jointly. *L. lactis* AA4, either inoculated singly or with *E. mundtii* CUGF08, maintained a stable high population level during the entire sprouting process (Figure 5.4), suggesting it can establish on alfalfa sprouts very easily and compete with other native microbiota for growth and nutrients. Interestingly, single inoculation of *E. mundtii* CUGF08 onto alfalfa seeds promoted the growth of *L. lactis* subsp. *lactis* (Figure 5.4). Native *L. lactis* subsp. *lactis* strains grew rapidly to 4-log CFU/g within 24 h and maintained the population steadily at approximately 5-log CFU/g. To exclude the possibility of contamination by *L. lactis* AA4, colonies on Alsan gar were randomly selected. PCR was performed to amplify their 16S rRNA gene to confirm that the *L. lactis* subsp. *lactis* on the non-AA4 inoculated sprouts were

not AA4 due to contamination. 16S rRNA gene sequencing and nucleotide homology analysis of selected colonies on Alsan agar revealed that the *L. lactis* subsp. *lactis* strains on Alsan agar were not *L. lactis* AA4. This implies that *L. lactis* subsp. *lactis* is an integral member of the native microbiota of alfalfa sprouts and *E. mundtii* CUGF08 may function as a selection factor for *L. lactis* subsp. *lactis* to outgrow other species by mechanisms of acid production or other unknown factors. Without *E. mundtii* CUGF08 as a selective pressure or a growth-promoting factor, *L. lactis* subsp. *lactis* did not grow to detectable levels during 4 days of sprouting, as seen with the control.

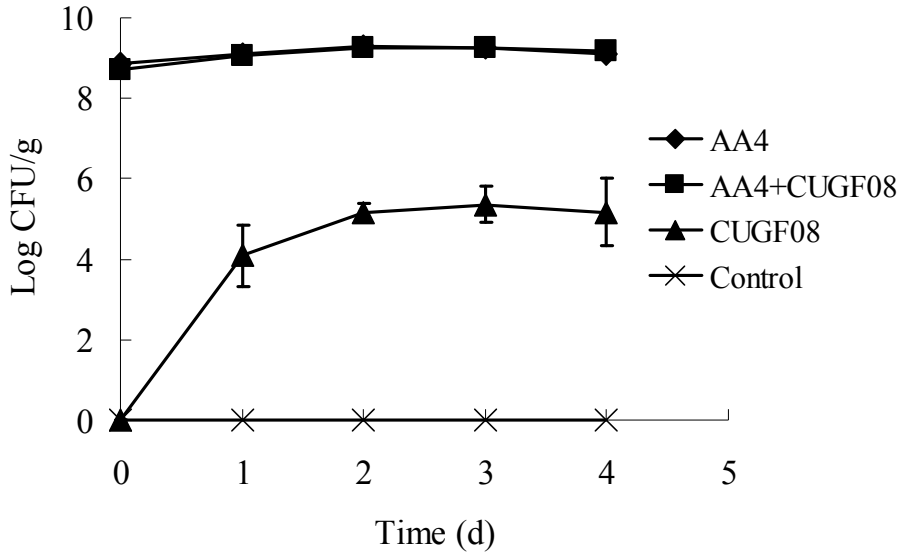

Figure 5.4. Growth of *L. lactis* AA4 on *Salmonella enterica*-contaminated alfalfa sprouts inoculated with *Lactococcus lactis* AA4, *Enterococcus mundtii* CUGF08, both, and none (control).

Unlike *L. lactis* AA4, *E. mundtii* CUGF08 did not maintain the level of inoculation to the end of sprouting when it was inoculated either singly or in combination with *L. lactis* AA4 (Figure 5.5). The level of *E. mundtii* CUGF08 gradually decreased by 1-log in 4 days of sprouting. Unlike *L. lactis* subsp. *lactis* whose growth was promoted

by *E. mundtii* CUGF08, growth of *Enterococcus* spp. was not promoted by single inoculation of *L. lactis* AA4. This implies that *Enterococcus* spp. may not be a ubiquitous member of the native microbiota of alfalfa sprouts or that the selection factors provided by *L. lactis* AA4 does not support the growth of *Enterococcus* spp. This hypothesis was evidenced by the fact that the population of *Enterococcus* spp. on sprouts co-inoculated with *L. lactis* AA4 and *E. mundtii* CUGF08 was significantly lower than that of *Enterococcus* spp. on sprouts that were inoculated solely with *E. mundtii* CUGF08 (Figure 5.5). There was no detectable growth of *Enterococcus* spp. on sprouts without inoculation of protective cultures.

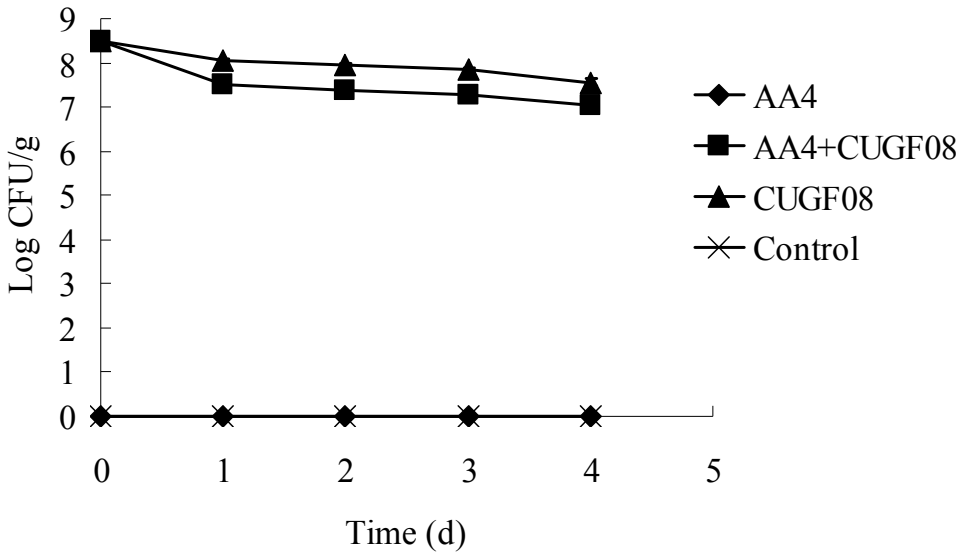

Figure 5.5. Growth of *Enterococcus* spp. on *Salmonella enterica*-contaminated alfalfa sprouts inoculated with *Lactococcus lactis* AA4, *Enterococcus mundtii* CUGF08, both, and none (control).

***In situ* bacteriocin activity and pH of finished sprouts.** There was no detectable bacteriocin activity of mundticin L or nisin Z in the finishing sprouts over the course of the sprouting process, as determined by the spot diffusion assay. However, this does not suggest that no bacteriocin was produced by the protective cultures since the

detection limit by the spot diffusion assay is not sufficiently sensitive. In addition, there could be many additional factors that influence the detection of bacteriocins, such as proteolytic enzymes in the samples that could have degraded bacteriocins prior to detection or interfering compounds contained in the sprout samples. The production of mundticin L should be constitutive due to the lack of three-component regulatory system in the genetic organization of mundticin L (7) although it was not detected in sprouts. Both mundticin L and nisin Z have little inhibitory activity against Gram-negative pathogens but they may have been involved in control of *L. monocytogenes* that was observed in the inoculated bioprotective culture samples.

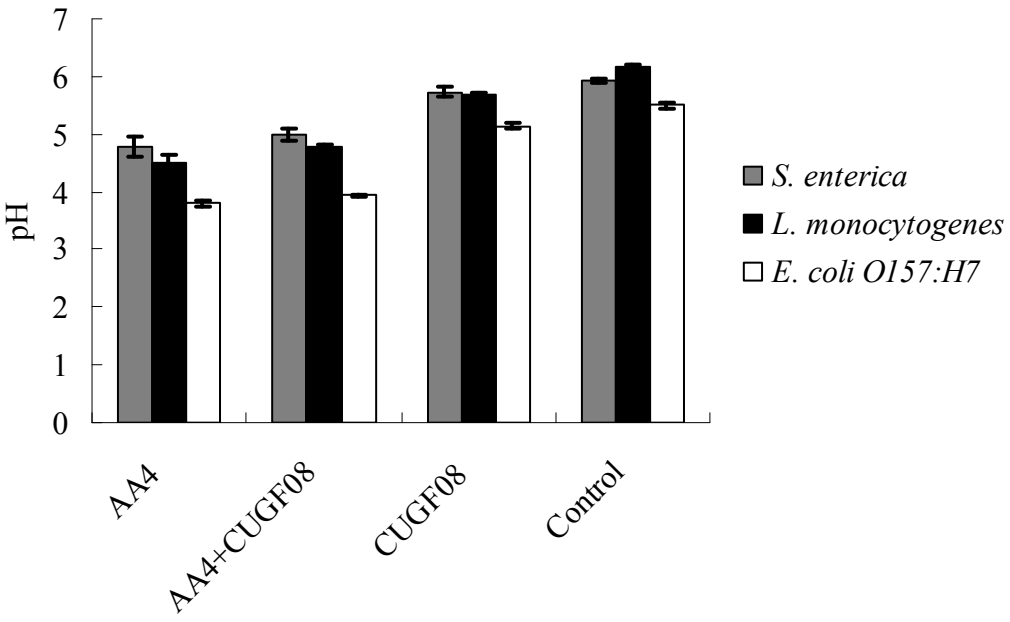

Figure 5.6. pH of finished sprouts contaminated with *S. enterica*, *E. coli* O157:H7 or *L. monocytogenes* and inoculated with *L. lactis* AA4, *L. lactis* AA4 and *E. mundtii* CUGF08, *E. mundtii* CUGF08 or no protective cultures (control).

The pH of finished sprouts was influenced by protective cultures regardless of the pathogens for pre-inoculation (Figure 5.6). *L. lactis* AA4 inoculated alone or co-inoculated with *E. mundtii* CUGF08 reduced the pH of sprouts to lower levels than the

samples inoculated with *E. mundtii* CUGF08 and the control. Sprouts without any inoculated protective cultures had highest pH. The pH of finished sprouts contributed at least partially to the difference in population of pathogens on sprouts while other factors that could be involved in antibiosis are largely unknown.

We have shown that protective cultures may be used to significantly control the growth of pathogens on sprouts. Although protective cultures did not inhibit the growth of *L. monocytogenes*, *S. enterica* and *E. coli* O157:H7 completely, the inoculation levels of pathogens were artificially higher compared to the natural contamination levels which could be as low as 1-6 pathogens per 100 g of seeds (10). Studies are currently being performed to challenge alfalfa seeds with pathogens levels comparable to those observed in seeds that have been responsible for foodborne illness outbreaks.

REFERENCES

1. **Alzoreky, N., and W. E. Sandine.** 1991. *Lactococcus* genus - a selective and differential agar medium. J. Food Sci. **56:**1729-1734.
2. **Ariefdjohan, M. W., P. E. Nelson, R. K. Singh, A. K. Bhunia, V. M. Balasubramaniam, and N. Singh.** 2004. Efficacy of high hydrostatic pressure treatment in reducing *Escherichia coli* O157 and *Listeria monocytogenes* in alfalfa seeds. J. Food Sci. **69:**M117-M120.
3. **Bennik, M. H., W. van Overbeek, E. J. Smid, and L. G. Gorris.** 1999. Biopreservation in modified atmosphere stored mungbean sprouts: the use of vegetable-associated bacteriocinogenic lactic acid bacteria to control the growth of *Listeria monocytogenes*. Lett. Appl. Microbiol. **28:**226-232.
4. **Bennik, M. H., B. Vanloo, R. Brasseur, L. G. Gorris, and E. J. Smid.** 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: full characterization and interaction with target organisms. Biochim. Biophys. Acta. **1373:**47-58.
5. **Charkowski, A. O., C. Z. Sarreal, and R. E. Mandrell.** 2001. Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. J. Food Prot. **64:**1292-1298.
6. **Feng, G., J. J. Churey, and R. W. Worobo.** 2007. Thermal inactivation of *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J. Food Prot. **70:**1698-1703.
7. **Feng, G., G. K. Guron, J. J. Churey, and R. W. Worobo.** 2009. Characterization of mundticin L, a class IIa anti-*Listeria* bacteriocin from *Enterococcus mundtii* CUGF08. Appl. Environ. Microbiol. **75:**5708-5713.
8. **Fett, W. F.** 2006. Inhibition of *Salmonella enterica* by plant-associated pseudomonads in vitro and on sprouting alfalfa seed. J. Food Prot. **69:**719-728.

9. **Hu, H., J. J. Churey, and R. W. Worobo.** 2004. Heat treatments to enhance the safety of mung bean seeds. J. Food Prot. **67:**1257-1260.
10. **National Advisory Committee on Microbiological Criteria for Foods.** 1999. Microbiological safety evaluations and recommendations on sprouted seeds. Int. J. Food Microbiol. **52:**123-153.
11. **Neetoo, H., T. Pizzolato, and H. Chen.** 2009. Elimination of *Escherichia coli* O157:H7 from alfalfa seeds through a combination of high hydrostatic pressure and mild heat. Appl. Environ. Microbiol. **75:**1901-1907.
12. **Neetoo, H., M. Ye, and H. Chen.** 2008. Potential application of high hydrostatic pressure to eliminate *Escherichia coli* O157:H7 on alfalfa sprouted seeds. Int. J. Food Microbiol. **128:**348-353.
13. **Palmai, M., and R. L. Buchanan.** 2002. Growth of *Listeria monocytogenes* during germination of alfalfa sprouts. Food Microbiol. **19:**195-200.
14. **Proctor, M. E., M. Hamacher, M. L. Tortorello, J. R. Archer, and J. P. Davis.** 2001. Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa sprouts grown from seeds pretreated with calcium hypochlorite. J. Clin. Microbiol. **39:**3461-3465.
15. **Schillinger, U., and F. K. Lucke.** 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. **55:**1901-1906.
16. **Veljovic, K., A. Terzic-Vidojevic, M. Vukasinovic, I. Strahinic, J. Begovic, J. Lozo, M. Ostojic, and L. Topisirovic.** 2007. Preliminary characterization of lactic acid bacteria isolated from Zlatar cheese. J. Appl. Microbiol. **103:**2142-2152.
17. **Wilderdyke, M. R., D. A. Smith, and M. M. Brashears.** 2004. Isolation, identification, and selection of lactic acid bacteria from alfalfa sprouts for competitive inhibition of foodborne pathogens. J. Food Prot. **67:**947-951.

CHAPTER 6

DISCUSSION, CONCLUSIONS, AND PROSPECTUS

DISCUSSION AND CONCLUSIONS

Two bacteriocin-producing lactic acid bacteria (LAB) isolated from alfalfa sprouts were characterized. *L. lactis* AA4 produces nisin Z while *E. mundtii* CUGF08 produces mundticin L, a newly identified class IIa bacteriocin that exhibits high antimicrobial activity against *Listeria* spp. and other genera (4). Mundticin L is composed of 43 amino acid with a YGNGX motif in its N-terminus. Unlike the homologous molecules such as mundticin ATO6/KS (1, 9) and enterocin CRL35 (11), the fifth amino acid of the YGNGX motif in mundticin L is leucine instead of valine. In addition, several different amino acids in the immunity protein and the ABC transporter make mundticin L a unique molecule.

The genetic determinants consist of three genes organized in two operons. The first operon contains a single gene encoding the mundticin L prepeptide while the second operon contains two genes encoding the ABC transporter and the immunity protein. This type of genetic organization represented by mundticin L, ATO6/KS and enterocin CRL35 is unique compared to other class IIa bacteriocins. In most cases, the structural and immunity genes are clustered in the same operon. However, these genes for mundticins are organized separately in two operons. This raises a question as to how transcription of these two genes is coordinated to ensure sufficient immunity levels to the mundticin bacteriocins.

Interestingly, the mundticin L producer was found to be deficient in immunity to its own bacteriocin as observed with agar diffusion assay. The gene for the immunity protein was heterologously expressed in sensitive strains to determine if this unusual phenotype was due to the function of its immunity protein. The expressed immunity

protein did not confer full protection to the sensitive strains, suggesting that the protein itself may not be sufficient for full immunity. Considering the fact that *E. mundtii* CUGF08 is able to grow to high cell density in liquid culture, additional unknown factors or additional immunity-related proteins might be involved in this phenotype. A genome-wide mutagenesis may elucidate the molecular mechanism or additional genes involved in this unusual immunity phenotype.

Alfalfa sprouts are the most commonly associated type of sprouts for foodborne illness outbreaks. The second part of this study was to challenge artificially contaminated alfalfa seeds with intervention strategies that would decontaminate sprouting seeds regardless of seed surface structures such as crevices and wrinkles where pathogens may hide inside and become inaccessible to surface sanitizers (2).

Mild heating and biocontrol using protective cultures are potential intervention methods. Unlike chemical sanitizers, mild heating can inactivate pathogens trapped in the seeds as well as pathogens on the surface. Mild heating has been shown to be effective in achieving 5-log reduction of *S. enterica* and *E. coli* O157:H7 on mung bean seeds (7). 5-log reduction of both pathogens on contaminated alfalfa seeds was observed in this study. The seeds can be treated at 55°C for up to 6 days without negatively affecting germination rates. However, alfalfa seeds can not tolerate more than 6 days of heating which results in decreased seed viability (germination) (3). Although mild heating was shown to be effective in achieving 5-log reduction in pathogens, post-processing contamination is still possible due to contaminated irrigation water and improper handling of seeds prior to sprouting or the finished sprouts. Food-grade lactic acid bacteria may be applied to alfalfa seeds to provide a sustainable antimicrobial hurdle not only prior to sprouting but also during the course of sprouting. Two bacteriocinogenic LAB strains isolated from alfalfa sprouts were studied for their bioprotective potential in enhancing the safety of contaminated alfalfa

seeds and sprouted seeds. Using LAB isolated from alfalfa sprouts has two advantages: it is more acceptable for consumers to include protective non-pathogenic cultures that are naturally occurring in sprouts; LAB from sprouts may be more adapted to the conditions in seeds and sprouts and have improved competitiveness with the rest of the sprout microbiota, compared to protective cultures from other non-sprout sources.

Nisin Z and mundticin L represent class I and IIa bacteriocins, respectively. They are two bacteriocin groups that are considered to be the most promising for application in foods as a means to enhance the safety and extend shelf life. Although their antibiosis against Gram-negative pathogens such as *S. enterica* and *E. coli* O157:H7 is limited, nisin Z and mundticin L have a defined activity against *L. monocytogenes*. In addition, these two bacteriocins may affect the microbial ecology of the diverse background microorganisms and as a result, may have an impact on Gram-negative pathogens. Due to the beneficial properties of LAB as potential probiotics, the use of LAB as protective cultures in plant-based foods has attracted an abundance of scientific interest. Sprouted seeds are an excellent food for challenge studies with protective cultures considering the diverse microbiota and their rapid growth during sprouting which may serve as a model for application of LAB in other foods.

In this study, both *L. lactis* AA4 and *E. mundtii* CUGF08 significantly controlled the growth of *L. monocytogenes* and *S. enterica* during sprouting. In general, *L. lactis* AA4 showed higher efficacy for the inhibition of *L. monocytogenes* and *S. enterica* compared to *E. mundtii* CUGF08. However, *E. mundtii* CUGF08 was more effective in controlling *E. coli* O157:H7 than *L. lactis* AA4. *L. lactis* is known as a strong acid producer compared to non-aciduric lactic acid bacteria that include *Enterococcus* spp. This was confirmed in these studies by the observed lower pH of finished sprouts inoculated with *L. lactis* AA4. However, *E. coli* O157:H7 is more acid resistant than *S.*

enterica and the mechanism of inhibition on *E. coli* O157:H7 by *E. mundtii* CUGF08 may be less related to acid production.

A significant synergistic effect of co-culture was not observed. Although both strains showed significant inhibition towards pathogens compared to the control (without protective cultures), pathogen growth was not completely controlled. This may be due to the fact that the inoculation level of pathogens on alfalfa seeds was artificially higher than that observed with "natural" contamination on commercial seeds. Studies are being performed to challenge alfalfa seeds inoculated with pathogen levels that are comparable to "natural" contamination levels.

PROSPECTUS

The author characterized the genetics and biochemical properties of mundticin L, a class IIa bacteriocin. The list of class IIa bacteriocins will continue to expand. This group of bacteriocins is the most promising after the approval of nisin by FDA as a food additive due to their high antimicrobial activity especially against *L. monocytogenes*, and stability over a wide range of pH and temperature conditions. Scientific efforts for the identification and characterization of new class IIa bacteriocins are ongoing. The relationship of bacteriocin structure and function along with their role in influencing complex microbial ecology will be an active area of research.

With the cost of nucleotide sequencing decreasing and more genomes of LAB being sequenced, genomics will accelerate the discovery of new bacteriocins. Genomics as a tool to discover new bacteriocins has advantages over phenotypic observations. For example, bacteriocins whose production is conditional, due to the variation in sensitivity of indicator strains, may be revealed by genome data mining. Also, related genes are more readily to be identified. However, it is difficult to locate potential bacteriocin genes due to their relatively small molecular size on the genome. Although

previous data for reported bacteriocins may provide clues and prediction models to facilitate genome data mining and bacteriocin characterization (5, 6), discovery of new bacteriocins by genomics tools is limited due to the lack of a universal model for identification of bacteriocin gene clusters and accurate annotation (10, 12). Genomics will by no means replace phenotypic screening for new bacteriocins.

In this study, an unusual phenotype of *E. mundtii* CUGF08 was observed. The mundticin L producer is deficient in immunity to its own bacteriocin as demonstrated by agar diffusion assay. This phenotype seems unique to *E. mundtii* strains producing mundticins. An investigation into the molecular mechanism of this unusual immunity phenotype may reveal new factors that have not been previously reported.

Two bacteriocinogenic LAB were studied for their potential as bioprotective cultures to enhance the safety of sprouts. They showed antibiosis against all three pathogens tested, although which LAB is more effective depends on the target pathogen of interest. Biological control of microorganisms on sprouted seeds is extremely challenging due to the growth-promoting environment and diverse microbiota that naturally exist on alfalfa seeds. Therefore, alfalfa sprouts may serve a model that could be used to determine the feasibility of protective cultures as a means to enhance the safety of other types of produce. This study suggests that biological control of pathogens may be an alternative strategy or at least an additional hurdle in combination with other decontamination methods.

Microbiology is advancing from the era of unicellularity to "multicellularity" for the 21st century. Scientific evidence has shown that individual microorganisms are coordinated by signaling molecules just like constituent cells in a eukaryotic tissue. While signaling molecules are acyl homoserine lactones in Gram-negative bacteria, cell-to-cell interaction in Gram-positive bacteria is mediated by small peptides (8). Bacteriocins are amid the context of the typical ecology and may play an important

role in intra-species and even inter-species interactions. Given the diversity of the microbiota on alfalfa seeds, the antibiosis of protective cultures against other microorganisms may be a result of more complex undiscovered factors other than just acid production. Scientific advances in this area are essential in understanding the mechanisms and may promote application of bioprotective cultures in a more easily defined manner.

With increasing market demand for minimally processed foods with “green” and “natural” preservatives, more studies will emerge in the area of bio-preservation using food-grade lactic acid bacteria that can protect foods from being contaminated while promote the health of consumers as probiotics.

REFERENCES

1. **Bennik, M. H., B. Vanloo, R. Brasseur, L. G. Gorris, and E. J. Smid.** 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: full characterization and interaction with target organisms. Biochim. Biophys. Acta. **1373:**47-58.
2. **Charkowski, A. O., C. Z. Sarreal, and R. E. Mandrell.** 2001. Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. J. Food Prot. **64:**1292-1298.
3. **Feng, G., J. J. Churey, and R. W. Worobo.** 2007. Thermal inactivation of *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J. Food Prot. **70:**1698-1703.
4. **Feng, G., G. K. Guron, J. J. Churey, and R. W. Worobo.** 2009. Characterization of mundticin L, a class IIa anti-*Listeria* bacteriocin from *Enterococcus mundtii* CUGF08. Appl. Environ. Microbiol. **75:**5708-5713.
5. **Hammami, R., A. Zouhir, J. Ben Hamida, and I. Fliss.** 2007. BACTIBASE: a new web-accessible database for bacteriocin characterization. BMC Microbiol. **7:**89.
6. **Hammami, R., A. Zouhir, C. L. Lay, J. Ben Hamida, and I. Fliss.** 2010. BACTIBASE second release: a database and tool platform for bacteriocin characterization. BMC Microbiol. **10:**22.
7. **Hu, H., J. J. Churey, and R. W. Worobo.** 2004. Heat treatments to enhance the safety of mung bean seeds. J. Food Prot. **67:**1257-1260.
8. **Irie, Y., and M. R. Parsek.** 2008. Quorum sensing and microbial biofilms. Curr. Top. Microbiol. Immunol. **322:**67-84.
9. **Kawamoto, S., J. Shima, R. Sato, T. Eguchi, S. Ohmomo, J. Shibato, N. Horikoshi, K. Takeshita, and T. Sameshima.** 2002. Biochemical and genetic

characterization of mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. Appl. Environ. Microbiol. **68:**3830-3840.

10. **Pfeiler, E. A., and T. R. Klaenhammer.** 2007. The genomics of lactic acid bacteria. Trends Microbiol. **15:**546-553.
11. **Saavedra, L., C. Minahk, A. P. de Ruiz Holgado, and F. Sesma.** 2004. Enhancement of the enterocin CRL35 activity by a synthetic peptide derived from the NH_2-terminal sequence. Antimicrob. Agents Chemother. **48:**2778-2781.
12. **Ventura, M., S. O'Flaherty, M. J. Claesson, F. Turroni, T. R. Klaenhammer, D. van Sinderen, and P. W. O'Toole.** 2009. Genome-scale analyses of health-promoting bacteria: probiogenomics. Nat. Rev. Microbiol. **7:**61-71.